Development of strategies for the prioritization of organic trace substances in water by effect-directed analysis

Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften
(Dr. rer. nat.)

Fakultät Naturwissenschaften

Universität Hohenheim

Institut für Lebensmittelchemie

vorgelegt von

Lena Stütz

aus Schwäbisch Gmünd

2020

Dekan:	Prof. Dr. Uwe Beifuß
1. berichtende Person, 1. Prüfer:	Prof. Dr. Wolfgang Schwack
2. berichtende Person, 2. Prüfer:	Prof. Dr. Walter Vetter
3. Prüfer:	Prof. Dr. Torsten C. Schmidt
Eingereicht am:	30.03.2020
Mündliche Prüfung am:	14.08.2020

Die vorliegende Arbeit wurde am 16. Juli 2020 von der Fakultät Naturwissenschaften der Universität Hohenheim als „Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften“ angenommen.

Acknowledgement

My sincere thanks go to all those who have contributed to the success of this work:

First of all, I would like to thank Prof. Dr. Wolfgang Schwack for the supervision of the work, for good and honest advice as well as for the support during the publication processes. I would like to thank Prof. Dr. Walter Vetter for taking over the co-report of the dissertation and Prof. Dr. Torsten C. Schmidt for his consent to be available as third examiner.

I would especially like to thank Dr. Wolfgang Schulz for his support on behalf of the Zweckverband Landeswasserversorgung. He gave me lot of advice and helped me with many good ideas.

My sincere thank goes to all my colleagues of the Laboratory for Operation Control and Research of Zweckverband Landeswasserversorgung for the excellent cooperation, the great working atmosphere and the support in all questions I had.

Furthermore, I would like to thank the students of the Aalen University of Applied Sciences and University of Hohenheim, who supported me in many respects and thus advanced my work. Finally, I want to thank the colleagues from the TZW: DVGW-Technologiezentrum Wasser for the good cooperation within the research project “WBA-BeReit”.

Table of contents

Abbreviation list

1D	One-dimensional
2D	Two-dimensional
AC	Activated carbon filtration
AChE	Acetylcholinesterase
AF	*Aliivibrio fischeri*
AMD	Automated multiple development
AOP	Adverse outcome pathway
AR	Androgen receptor
BS	*Bacillus subtilis*
CAS	Chemical Abstract Service
CHO-cells	Chinese ovary hamster-cells
DDT	Dichlorodiphenyltrichloroethane
DIN	German Institute for Standardization
DNA	Deoxyribonucleic acid
DR	Dioxin response
DW	Drinking water
EDA	Effect-directed analysis
ER-α	Estrogen receptor-α
ER-CALUX®	Estrogen receptor-chemically activated luciferase expression®
ESI	Electrospray ionization
Fc	Fold change
FEA	Federal Environmental Agency
GC	Gas chromatography
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography

HRMS	High-resolution mass spectrometry
ISO	International Organization for Standardization
LC	Liquid chromatography
MS	Mass spectrometry
MS/MS	Tandem-mass spectrometry
MUG	4-Methylumbelliferyl-ß-D-galactopyranoside
MTT	3-[4,5-Dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate hydrogen
NP	Normal phase
NT	Non-target
NTS	Non-target screening
O_3	Ozonation
ONPG	O-Nitrophenyl-β-D-galactopyranoside
PAH	Polycyclic aromatic hydrocarbons
pYES	Planar yeast estrogen screen
QSAR	Quantitative structure-activity relationship
RP	Reversed phase
RW	Raw water
SPE	Solid phase extraction
TLC	Thin-layer chromatography
WBA	Wirkungsbezogene Analytik
YES	Yeast estrogen screen

Glossary

Ames assay	*In-vitro* bioassay based on genetically modified bacterial *Salmonella* strains. It is used to detect mutagenic effects and was developed by Bruce Ames in 1975.
DR-LUC assay	Reporter gene assay for the detection of dioxin-like effects. Dioxin response (DR) is mediated by the *luc*-gene coding for the enzyme luciferase. Luciferase catalyses light emission of the substrate luciferin.
GeneBLAzer assay	By binding of estrogenic substances to the estrogen receptor-α (ER-α) in the ER-GeneBLAzer assay, a signalling cascade starts in which the ß-lactamase is expressed by the *bla*-gene. The ß-lactamase cleaves a substrate whose change in fluorescence can be detected.
H295R assay	*In-vitro* bioassay with human H295R-adenocarcinoma cell line for the detection of non-receptor bound endocrine effects.
HeLa-9903	Human, genetically modified adenocarcinoma cell line originally taken from Mrs. Henrietta Lacks in 1951.
LacZ-reporter gene	Gene of lactose-operon (*lac*-operon) in bacteria encoding the enzyme ß-galactosidase, which cleaves lactose to glucose and galactose. Additional to the natural substrate lactose, the enzyme also cleaves color substrates to the dye and galactose, such that the dye concentration can be determined photometrical.
MELN	Estrogen-sensitive human breast cancer cell line containing a luciferase reporter gene induced through binding of compounds to the ER-α.
Metabolic activation	Many environmental substances are chemically altered in the liver of animals and humans by xenobiotic metabolism and thus acquire a toxicological effect. Bacterial cells usually do not possess enzymes of xenobiotic metabolism and are called metabolically not-competent. In order to simulate the processes of xenobiotic metabolism, exogenous enzymes are added to the bacterial cells.
Micronucleus assay	Assay for the detection of chromosome damage and damage to the spindle apparatus of a cell due to genotoxic effects. The determination of genotoxicity is carried out by the formation of micronuclei during cell division that contain damaged chromosome fragments.

P53-CALUX® assay	Reporter gene assay for the detection of genotoxic effects. P53 (protein, 53 kDa) transcription factor regulates gene expression after DNA damage. In the assay, activation of the p53-pathway leads to expression of luciferase. Luciferase catalyses light emission of the substrate luciferin.
SOS-repair system	The SOS-response repairs DNA in damaged bacterial cells before the cell cycle continues.
Umu assay	*In-vitro* bioassay for the detection of genotoxic effects to a genetically modified *Salmonella* strain. The assay is mediated by the bacterial cell's SOS-repair system, which contains an *umuC*-gene that gave the assay its name.
S. cerevisiae BJ3505	Genetically modified *Saccharomyces* yeast cell strain that carries the gene for the human ER-α. If estrogenic substances bind to the receptor, it triggers the expression *lacZ*-gene coding for ß-galactosidase.
S. typhimurium TA98	Histidine-deficient mutant *Salmonella* strain in the Ames assay, for the detection of base pair substitutions.
S. typhimurium TA100	Histidine-deficient mutant *Salmonella* strain in the Ames assay, which is used to detect frameshift mutations. A frameshift mutation is a change in the genetic reading frame of the DNA sequence.
WBA-BeReit	Acronym for the research project: Wirkungsbezogene Analytik als neuer Ansatz zur orientierenden Bewertung organischer Spurenstoffe in Rohwasserressourcen zur Trinkwassergewinnung und bei Aufbereitungsprozessen.

Preliminary remarks

This dissertation was carried out between July 2016 and February 2020. Supervisor was Prof. Dr. Wolfgang Schwack, Institute of Food Chemistry, University of Hohenheim, Germany. Practical work was performed at the Laboratory for Operation Control and Research at Zweckverband Landeswasserversorgung in Langenau, Germany.

Parts of this work have already been published in international peer-reviewed journals, or were presented at national conferences as oral or poster presentations:

Full publications

(1) L. Stütz, S.C. Weiss, W. Schulz, W. Schwack, R. Winzenbacher, Selective two-dimensional effect-directed analysis with TLC, *Journal of Chromatography A* 1524 (2017) 273-282.

(2) L. Stütz, P. Leitner, W. Schulz, R. Winzenbacher, Identification of genotoxic transformation products by effect-directed analysis with high-performance thin-layer chromatography and non-target screening, *Journal of Planar Chromatography - Modern TLC* 32 (2019) 3, 173-182.

(3) L. Stütz, W. Schulz, R. Winzenbacher, Identification of acetylcholinesterase inhibitors in water by combining two-dimensional HPTLC and high-resolution mass spectrometry, *Journal of Chromatography A*, 1624 (2020) 461239.

Oral presentations

(1) L. Stütz, Zweidimensionale Wirkungsbezogene Analytik mit Hochleistungsdünnschicht-chromatographie, 27. *Doktorandenseminar des Arbeitskreises Separation Science*, January 8 - 10 (2017) Hohenroda, Germany.

(2) L. Stütz, W. Schulz, R. Winzenbacher, Wirkungsbezogenes Monitoring zur Priorisierung relevanter Spurenstoffe bei der Trinkwasseraufbereitung, *Mülheimer Wasseranalytisches Seminar*, September 12 - 13 (2018) Mülheim, Germany.

(3) L. Stütz, Wirkungsbezogene Analytik zur Priorisierung und Erstbewertung organischer Spurenstoffe in der aquatischen Umwelt, *Fachkolloquium Trinkwasser*, September 2 (2019) Gelsenkirchen, Germany.

Poster presentations with short lecture

(1) L. Stütz, D. Esslinger, W. Schulz, W. Schwack, R. Winzenbacher, Wirkungsbezogenes Monitoring zur Priorisierung von Spurenstoffen in Roh- und Trinkwasserproben, *Annual Meeting of the Wasserchemische Gesellschaft*, Mai 7 - 9 (2018) Papenburg, Germany.

Poster presentations

(1) L. Stütz, S.C. Weiss, W. Schulz, W. Schwack, R. Winzenbacher, Selective two-dimensional effect-directed analysis with TLC, *International symposium for high-performance thin-layer chromatography*, July 4 - 8 (2017) Berlin, Germany.

Miscellaneous

(1) L. Stütz, S.C. Weiss, Two-dimensional effect-directed analysis of a surface water sample, *CAMAG Bibliography Service* 121 (2018), 2-4.

(2) W. Schulz, O. Happel, L. Stütz, B. Schmutz, WBA-BeReit - Wirkungsbezogene Analytik als neuer Ansatz zur orientierenden Bewertung organischer Spurenstoffe in Rohwasserressourcen zur Trinkwassergewinnung und bei Aufbereitungsprozessen, Final report, DVGW-Research project, *https://www.dvgw.de/themen/forschung-und-innovation/forschungsprojekte/dvgw-forschungsprojekt-wba-bereit* (2019).

(3) L. Stütz, W. Schulz, R. Winzenbacher, O. Happel, B. Schmutz, M. Scheurer, Wirkungsbezogene Analytik in der Trinkwassergewinnung, *DVGW energie*│*wasser-praxis* 2 (2020), 46-51.

Contributions

The contributions and responsibilities of the persons named in the full publications were as follows:

Lena Stütz performed all essential practical work of the dissertation. She planned the experiments and carried out the practical work. She evaluated the data, interpreted the results and wrote the manuscripts for publication.

Wolfgang Schwack supervised the work on the part of the University of Hohenheim. He corrected the manuscripts for publication and accepted the formal organization of the dissertation.

Wolfgang Schulz supervised the work on behalf of the Zweckverband Landeswasserversorgung. He advised in finding ideas and supported in analytical issues.

Rudi Winzenbacher initiated the scientific question of the work and organized the financial support of the research project "WBA-BeReit". He corrected the manuscripts and gave advice on how to proceed.

Stefan Weiss implemented and optimized the acetylcholinesterase inhibition assay and thus made a contribution to the development of the selective two-dimensional effect-directed analysis of Publication 1.

Patricia Leitner implemented the umu assay for measurement of genotoxicity at Zweckverband Landeswasserversorgung and helped with the practical execution of the bioassay during preparation of Publication 2.

1 Summary

The protection of the aquatic environment and the supply of clean drinking water to people all over the world are central challenges of our time. Monitoring of the aquatic environment and the input of anthropogenic trace substances into it is therefore very important. However, since aquatic environmental samples often consist of complex substance mixtures, their characterization and evaluation is very demanding. By using generic target analysis methods, selected known anthropogenic trace substances can be detected and quantified very sensitively. For the detection of previously unknown substances, non-target analysis methods have been increasingly used in recent years. However, these methods do not provide information on the relevance of the anthropogenic trace substances occurring in water. In this context, especially all those trace substances are regarded as relevant from which a harmful effect on humans or water organisms is to be expected. For the detection of such effective substances, effect-directed analysis (EDA) can be used, which specifically detect effects such as neurotoxicity or genotoxicity. In EDA, a bioassay is combined with a fractionation method and subsequent chemical analysis, the aim being to identify the bioactive substance. The EDA detects the effect of individual substances or substance groups in a sample with the respective endpoint without the need to know the effective substances. The separation method used in this work is high-performance thin-layer chromatography (HPTLC). After chromatography, the bioassay is performed directly on the HPTLC plate. If an effective zone appears in the bioassay, a prioritization strategy is used to clarify the identity of the substance.

Due to the complex aquatic samples, a large number of different substances in a zone must still be expected despite the applied HPTLC separation, which makes it difficult to identify the effective substance. Therefore, a strategy to simplify the identification of effective substances should be developed. The aim was to reduce the complexity by multidimensional separation in such a way that chemical analysis can be used to prioritize to a few candidates in the effective fraction.

In the first part of the work, a selective two-dimensional HPTLC separation was developed to reduce the number of substances in a bioactive zone. After the first separation dimension (1D) the acetylcholinesterase inhibition assay (AChE assay) was performed and afterwards only the effective zones were extracted from the HPTLC plate. The selected effective zones were separated in a second separation dimension (2D) and the bioassay was performed again. Here, a retardation factor-adapted separation with several mobile phases was applied in the 2D. Depending on the retardation factor of the effective zone in the 1D, a mobile phase with optimized eluent strength was used in the 2D. This procedure was intended to achieve an optimal separation of the samples and thus a reduction of the complexity of the effective zones. With this 2D separation, the peak capacity could be increased by a factor of 7 compared to a 1D HPTLC gradient development.

If real water samples are examined for their effects, an additional structural elucidation must be carried out to clearly identify the unknown bioactive substances. In this work, the developed 2D EDA was therefore connected to a high-performance liquid chromatography (HPLC) with high-resolution mass spectrometry (HRMS) and a non-target screening (NTS) was performed. Using three water samples (drinking water, surface water and purified sewage water) spiked with six effective substances, it was

shown that the developed strategy is suitable for the identification of effective substances and that these can be recovered despite repeated extraction. When applying the developed methodology to real samples, it was also possible to assign and quantify the detected effect in several waters to the substance lumichrome and to linear alkylbenzene sulfonates.

Genotoxicity is a crucial endpoint for the effect assessment of water samples. However, this endpoint with metabolic activation cannot yet be performed directly on the HPTLC plate. Since many of the genotoxic substances have an indirect genotoxic effect, i.e. they only acquire their activity after metabolic activation; this endpoint was investigated in the present work with the umu assay in the microtiter plate. However, separation with HPTLC, subsequent extraction of effective zones and non-target analysis of the extracts, should also be performed for this assay. Therefore the umu assay in the microtiter plate was integrated into the existing EDA-with-HPTLC concept.

In laboratory experiments, sodium hypochlorite was added to the drug metformin in order to simulate the behavior of the substance during water treatment (chlorination). The metformin sample treated with hypochlorite was examined with the umu assay and a genotoxic effect was detected. After HPTLC separation of the chlorinated sample, zones were extracted over the entire retardation range. When the extracted zones were examined with the umu assay, the genotoxic effect could be clearly assigned to one fraction. Using high-resolution mass spectrometry, the genotoxic effect could be assigned to an already known transformation product of metformin. The HPTLC separation and extraction of the zones from the plate led to a reduction of the possible effective candidate masses by a factor of 10 and thus to a clear prioritization in HRMS analysis.

In the context of the present work, the project "WBA-BeReit" was also worked on, with the aim of showing the possibilities and the significance of the EDA for drinking water treatment. In the project, raw and drinking waters of different water suppliers from Germany were analyzed and the results were compared. Clear effect changes during the treatment of raw to drinking water were shown, but also when comparing the individual raw waters. Thus, it was possible to successfully use the EDA for an orienting evaluation of different treatment processes with regard to the change of effects during the treatment process of drinking water.

2 Zusammenfassung

Der Schutz der aquatischen Umwelt und die Versorgung der Menschen auf der ganzen Welt mit sauberem Trinkwasser sind zentrale Herausforderungen unserer Zeit. Daher ist die Überwachung der aquatischen Umwelt und des Eintrags von anthropogenen Spurenstoffen in diese von wichtiger Bedeutung. Da aquatische Umweltproben jedoch oft aus komplexen Stoffgemischen bestehen, ist deren Charakterisierung und Bewertung sehr anspruchsvoll. Durch den Einsatz klassischer Target-Analysemethoden können ausgewählte, bekannte anthropogene Spurenstoffe sehr empfindlich detektiert und quantifiziert werden. Für die Detektion bislang unbekannter Substanzen werden in den letzten Jahren vermehrt Non-Target-Analysemethoden eingesetzt. Allerdings liefern diese Methoden keine Aussagen zur Relevanz der im Wasser vorkommenden anthropogenen Spurenstoffe. Als relevant gelten in diesem Zusammenhang vor allem all jene Spurenstoffe, von denen eine schädliche Wirkung auf den Menschen oder im Wasser lebende Organismen zu erwarten ist. Zur Detektion solcher wirkender Substanzen kann die Wirkungsbezogene Analytik (WBA) verwendet werden, die gezielt Wirkungen wie zum Beispiel Neurotoxizität oder Gentoxizität detektiert. Bei der WBA wird ein Bioassay mit einer Fraktionierungsmethode und anschließender chemischer Analyse kombiniert, wobei das Ziel die Identifizierung der bioaktiven Substanz ist. Die WBA detektiert die Wirkung von einzelnen Substanzen bzw. Substanzgruppen in einer Probe mit dem jeweiligen Endpunkt, ohne dass dafür die wirkenden Substanzen bekannt sein müssen. Als Fraktionierungsmethode wurde in dieser Arbeit die Hochleistungsdünnschichtchromatographie (HPTLC) angewandt. Nach der Chromatographie wird der Bioassay direkt auf der HPTLC-Platte durchgeführt. Tritt im Bioassay eine wirkende Zone auf, so soll mithilfe einer Priorisierungsstrategie die Identität der Substanz aufgeklärt werden.

Aufgrund der komplexen aquatischen Proben muss trotz der angewandten HPTLC-Trennung in einer wirkenden Zone noch immer mit einer Vielzahl verschiedener Substanzen gerechnet werden, die eine Identifizierung der wirkenden Substanz erschweren. Es sollte aus diesem Grund eine Strategie zur Vereinfachung der Identifizierung von wirkenden Substanzen entwickelt werden. Ziel war es, die Komplexität durch mehrdimensionale Trennung so zu verringern, dass mithilfe der chemischen Analyse auf wenige Kandidaten in der wirkenden Zone priorisiert werden kann.

Im ersten Teil der Arbeit wurde zur Reduktion der Anzahl von Substanzen in einer bioaktiven Zone eine selektive zweidimensionale HPTLC-Trennung entwickelt. Nach der ersten Trenndimension (1D) wurde der Acetylcholinesterase-Hemmtest (AChE-Assay) durchgeführt und anschließend lediglich die wirkenden Zonen von der HPTLC-Platte extrahiert. Die ausgewählten wirkenden Zonen wurden in einer zweiten Trenndimension (2D) nochmals aufgetrennt und abermals der Bioassay durchgeführt. Hierbei kam in der 2D eine Retardationsfaktor-angepasste Trennung mit mehreren Fließmitteln zum Einsatz. Je nach dem Retardationsfaktor der wirkenden Zone in der 1D wurde in der 2D ein Fließmittel mit optimierter Elutionskraft verwendet. Mit diesem Vorgehen sollte eine optimale Auftrennung der Proben und damit eine Reduktion der Komplexität der wirkenden Zonen erreicht werden. Die Peakkapazität konnte durch diese 2D-Trennung im Vergleich zu einer 1D-HPTLC-Gradientenentwicklung um den Faktor 7 gesteigert werden.

Wenn reale Wasserproben wirkungsbezogenen untersucht werden, muss für eine eindeutige Identifizierung der unbekannten bioaktiven Substanzen zusätzlich eine Strukturaufklärung durchgeführt werden. In dieser Arbeit wurde deshalb die entwickelte 2D-WBA an eine Hochleistungsflüssigkeitschromatographie (HPLC) mit hochauflösender Massenspektrometrie (HRMS) angebunden und ein Non-Target-Screening (NTS) durchgeführt. Am Beispiel dreier mit sechs wirkenden Substanzen dotierter Wasserproben (Trinkwasser, Oberflächenwasser und gereinigtes Abwasser) konnte gezeigt werden, dass sich die entwickelte Strategie für die Identifizierung wirkender Substanzen eignet und dass diese trotz mehrmaliger Extraktion wiedergefunden werden können. Bei der Anwendung der entwickelten Methodik auf Realproben gelang es zudem, in mehreren Wässern die detektierte Wirkung der Substanz Lumichrome sowie linearen Alkylbenzolsulfonaten zuzuordnen und zu quantifizieren.

Für die Beurteilung der Wirkungen von Wasserproben ist die Gentoxizität ein entscheidender Endpunkt. Allerdings kann dieser Endpunkt mit metabolischer Aktivierung bislang nicht direkt auf der HPTLC-Platte durchgeführt werden. Da jedoch viele der gentoxischen Substanzen indirekt gentoxisch wirken, ihre Aktivität also erst nach der metabolischen Aktivierung erlangen, wurde dieser Endpunkt im Rahmen der vorliegenden Arbeit mit dem umu-Assay in der Mikrotiterplatte untersucht. Jedoch sollten auch für diesen Assay eine Fraktionierung mit der HPTLC, eine anschließende Extraktion wirkender Zonen und Non-Target-Analyse der Extrakte erfolgen. Dazu wurde der umu-Assay in der Mikrotiterplatte in das bestehende WBA-mit-HPTLC-Konzept integriert.

Im Laborexperiment wurde das Arzneimittel Metformin mit Natriumhypochlorit versetzt, um damit das Verhalten der Substanz während der Wasseraufbereitung (Chlorung) nachzustellen. Die mit Hypochlorit umgesetzte Metforminprobe wurde mit dem umu-Assay untersucht, wobei eine gentoxische Wirkung detektiert werden konnte. Nach der HPTLC-Trennung der gechlorten Probe wurden anschließend Zonen über den gesamten Retardationsbereich extrahiert. Bei der Untersuchung der extrahierten Zonen mit dem umu-Assay konnte der gentoxische Effekt eindeutig einer Fraktion zugeordnet werden. Mittels hochauflösender Massenspektrometrie gelang es, den gentoxischen Effekt einem bereits beschriebenen Transformationsprodukt von Metformin nachzuweisen. Die HPTLC-Trennung und Extraktion der Zonen von der Platte führte zu einer Reduktion der möglichen wirkenden Kandidatenmassen um den Faktor 10 und somit zu einer deutlichen Priorisierung bei der HRMS-Analyse.

Im Rahmen der vorliegenden Arbeit wurde zudem das Projekt „WBA-BeReit“ bearbeitet, mit dem Ziel, die Möglichkeiten sowie die Aussagekraft der WBA für die Trinkwasseraufbereitung aufzuzeigen. Im Projekt wurden u.a. Roh- und Trinkwässer verschiedener Wasserversorger aus Deutschland analysiert und die Ergebnisse verglichen. Es zeigten sich deutliche Wirkungsveränderungen durch die Aufbereitung von Roh- zum Trinkwasser, aber auch beim Vergleich der einzelnen Rohwässer. So war es möglich, die WBA erfolgreich für eine orientierende Bewertung verschiedener Aufbereitungsverfahren hinsichtlich der Veränderung der Wirkungen über den Aufbereitungsprozess von Trinkwasser hinweg einzusetzen.

3 Aims and scope

In this thesis, EDA was applied to detect bioactive substances in different water samples. Since not all of the ingredients in water samples can be identified and evaluated with the analytical methods known to date, effect detection should be used to prioritise the relevant substances, i.e., those that cause a damaging effect to humans and/or to the environment. Various *in-vivo* and *in-vitro* bioassays are currently available to assess the effects of such substances. However, due to the numerous ingredients in aquatic samples, the identification of effective substances still poses a major challenge. For this reason, a strategy should be developed in this work to **facilitate the identification of bioactive substances** in water. As a result of identification, measures could be taken to reduce or even prevent the introduction of bioactive substances in the aquatic environment.

One reason for failing identification attempts in EDA is the complexity of the samples, such that a large number of different substances still occur in an effective fraction. The aim of the first part of the work was therefore to **reduce the number of substances in a fraction by increasing the peak capacity with two-dimensional HPTLC separation**. A selective approach should be developed, in which only the effective zones are transferred into 2D (**Publication 1**). For identification of the bioactive compounds, the developed 2D EDA approach should be combined with high-resolution mass spectrometry and a **prioritization strategy** was to be developed. The number of signals that could be responsible for the effect should be further reduced by the formation of intersections of the sample and the extracted zones of the 1D and 2D. Prioritisation to a few signals should facilitate the identification of the effective substance (**Publication 3**).

In order to cover a broad effect spectrum, the endpoint genotoxicity should be added to the existing EDA with HPTLC concept in this thesis. The genotoxicity is of particular importance for water quality monitoring, as it results in very low health-related indication values of 0.1 µg/L (0.01 µg/L for genotoxic substances with relevant metabolism) in drinking water. Due to the fact that the metabolic activation of indirect genotoxic substances cannot be performed on the HPTLC plate yet, the umu assay for the detection of genotoxic effects should be applied in the microtiter plate. The aim of the current work was the development of a **workflow for prior HPTLC separation to the umu assay in the microtiter plate and combination with HRMS**, such that prioritization and identification of genotoxic substances gets possible with the EDA with HPTLC concept (**Publication 2**).

After the method development in the first part of the work, within the research project „WBA-BeReit“, a real world application of EDA was performed. It should be investigated, if EDA can be used for the **orienting evaluation of organic trace substances in raw water resources for drinking water extraction and treatment processes**. For this purpose an initial monitoring approach for raw and drinking water had to be developed and different treatment processes were compared.

4 Introduction

4.1 Organic trace substances in the aquatic environment

Far more than 100 million substances are registered by the Chemical Abstract Service (CAS) of the American Chemical Society up to 2019, and more than 100 000 were estimated to be in use presently [1]. Every year about 500 to 1000 new chemicals are synthesized [2]. These substances include, for example, pharmaceuticals, pesticides, industrial chemicals or cosmetic products, which find numerous applications in daily use and can thus, end up in the aquatic environment and subsequently also in raw water resources used for drinking water production (Table 1).

Table 1: Examples for organic pollutants in the aquatic environment [3,4]

Origin/Usage	**Class**	**Example groups/substances**
Industrial chemicals	Solvents	Tetrachloromethane
	Intermediates	Methyl-t-butylether
	Petrochemicals	BTEX (benzene, toluene, ethylbenzene, xylenes)
	Plasticizers	Phthalates
	Lubricants	Polychlorinated biphenyls
	Flame retardants	Triphenyl phosphate
Consumer products	Surfactants	Sodium dodecylbenzenesulfonate
	Pharmaceuticals	Metformin, Antibiotics
	Hormones	17α-Ethinylestradiol
	Personal care products	UV-filters
Biocides	Pesticides	Atrazine, DDT
	Non-agricultural biocides	Triclosan
Natural chemicals	Taste and odor compounds	Geosmin
	Cyanotoxins	Microcystins, Anatoxin
	Human hormones	17ß-Estradiol, Testosterone
Transformation products	formed from all the above through transformation processes	Desethylatrazine, Guanyl urea

Anthropogenic substances can enter the aquatic environment in different ways, such as direct sources like wastewater treatment plants or industrial processes. Other entry paths are non-point sources like agriculture or surface runoffs. The substances can be unintentionally released into the air, water or soil via diffuse inputs or accidents, or they can be deliberately released into the ecosystem, as in the case of pesticides. The different environmental areas are linked by transport and transfer processes such that the substances were distributed [2]. Additionally, the substances are further transformed to different transformation products.

Transformation processes can take place directly in humans or animals, as is desired, with many drugs. Transformations in the environment and during water treatment processes, like ozonation or chlorina-

tion cause additional transformation products. The vast majority of them are lesser persistent, bioaccumulative and toxic than their starting substances, but there are also some exceptions. For example, there is nonylphenol, which is more persistent and bioaccumulative than the precursor substance nonylphenol polyethoxylate and moreover causes estrogenic effects [5]. Substances and their transformation products are very diverse and many of the transformation products are currently not known. In addition, frequently nothing is known about their toxicity to the ecosystem or humans.

For water quality assessment, however, the (eco)toxicological effects of the substances occurring is of particular importance, as they pose a potential risk to animal and human health. For this reason, bioanalytical methods for the detection of toxic effects have been increasingly used in recent years [3]. By using bioassays, which are able to detect different endpoints, a broad effect spectrum can be covered. However, if the effective substance(s) in an aquatic sample are to be identified, fractionation and chemical analysis are required in addition to biotesting. Then the method is named effect-directed analysis (EDA).

4.2 Effect-directed analysis

EDA was developed to identify the major toxicants in complex environmental mixtures, which pose a risk to the environment or to human health. There are a lot of different substances in the environment, but often only a few key substances are responsible for causing an effect and thus for the possible existing risk. Therefore, EDA combines a fractionation with biological testing and chemical analysis with the aim to identify the bioactive substances (Figure 1) [6]. But it is also possible to compare different samples with EDA without knowing the identity of the effective substances. For example, the effects of samples from different seasons or sampling sites can be compared. Such an effect-directed consideration of samples is called bioprofiling [7-9].

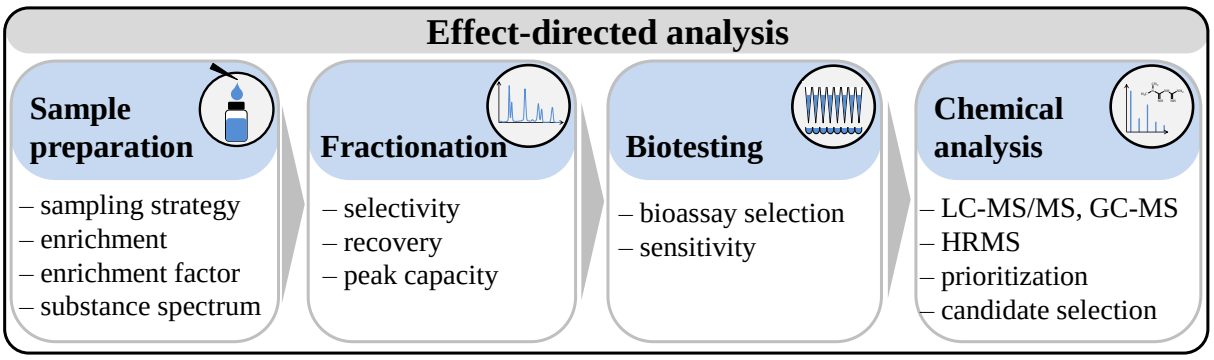


Figure 1: General workflow of effect-directed analysis, modified from [10].

The core of EDA is the biotesting, where samples are investigated regarding their toxicity to selected endpoints. If a sample shows an effect in the bioassay, EDA enables the separation of the sample into several fractions, which could be investigated with the biotesting again. In this work, HPTLC was used as a fractionation method for EDA. In this particular case, the bioassay is performed directly on the HPTLC plate and is not applied first without separation, but always after the chromatography. In the simplest case, the effect can be assigned to one fraction of the sample. So, the fractionation reduces the complexity of the sample and additionally delivers information of the physicochemical properties of possible toxicants. These can be determined by the retardation factor of the effective compounds,

but also by its absorption or fluorescence. The effective HPTLC zone is then further investigated with chemical analysis to identify the effective compound.

4.2.1 Sample preparation

Water samples to be investigated with EDA can differ widely, from treated sewage, surface or ground water up to drinking water. But the concentration of organic trace substances contained, generally is under the µg/L-range and so with few exceptions lies under the detection limit of the bioassays, such that sample enrichment becomes necessary [11]. As enrichment method for biotesting, solid phase extraction (SPE) is frequently used [3,12,13]. Depending on the load of the sample with organic trace compounds and matrix, the enrichment factor can be variably adjusted, whereby generally it ranges between 10 up to 10 000 [14]. Such flexible enrichment enables the detection of effects, even if the substances are only present in minute traces. But sample enrichment also poses challenges that should be considered [10]:

- **Toxicity blanks:** They may occur if impurities enter the sample during the extraction process. Therefore, a blank extract is always required that reflects the conditions of the extraction process. For example, a blank of the SPE cartridge helps to detect contamination of the solid phase material or the solvents used.
- **Detectable substances:** It has to be considered, that an extraction procedure will only gather parts of the substances contained in the sample. Therefore often multi-layer SPE cartridges are used for biotesting, to gather a broad range of different substance classes. For example, C18 solid phase materials are combined with special polymer phases that enable the targeted enrichment of polar substances or of acidic resp. basic substances [15]. Still, the enriched sample must not be representative for the original water sample, because there will always be substances, like metals or very polar organic substances, which could only be enriched partly or are completely lost during the enrichment.
- **Matrix effects:** There are matrix compounds, which are also enriched with the used enrichment method. These compounds generally are natural ones, like humic and fulvic acids and they may interact with the bioassay and cause unspecific effects [16]. But they also may bind substances contained in the samples and decrease their bioavailability for the bioassay. Such matrix effects increase with increasing enrichment factor. Clean-ups may counteract, but they cause the additional risk of infiltration of blank toxicity and loss of substances.

In addition to enrichment with SPE, other enrichment methods, like vacuum concentration or freeze-drying can also be used for EDA. By using these methods, the substance spectrum to be covered can be extended. Vacuum concentration and freeze-drying are well suited for the enrichment of polar and ionic substances which can only be enriched to a limited extent by SPE [17,18]. In the research project “WBA-BeReit” that was processed in this thesis, very good recoveries for selected anions, cations and carboxylic acids could be achieved with these methods. However, the salts contained in the samples were also enriched and influenced the applied chromatography. It was also shown that the achievable enrichment factors of approx. 50 - 100 limit the application of these methods to water samples with a higher content of effective substances [19].

4.2.2 Fractionation

The fractionation step in EDA is intended to reduce the complexity of a sample and thus the number of substances in a fraction that could possibly be responsible for the effect. By separating non-effective fractions from effective fractions, the following identification strategy can be aligned to those fractions that cause an effect in the bioassay. In order to develop an optimal fractionation method for the addressed separation problem, the following criteria must be observed [10]:

- **Recovery of toxicity:** It must be ensured that toxicity is recovered after fractionation. For this purpose, the fractions can be combined and their effect compared with the effect of the original sample.
- **No toxicity of the blank:** Toxicity blank values, which result from the solvent used or from the fractionation process, must be controlled and largely prevented.
- **Selectivity and orthogonality of separation steps:** The selected fractionation of the sample must show sufficient selectivity for the separation problem to be solved. Orthogonal multi-dimensional fractionations can be used to increase the separation efficiency [20].
- **Information for toxicant identification:** Fractionation can be used to obtain additional information about the effective substance, such as the estimation of log K_{ow} values. Using a quantitative structure-activity relationship (QSAR)-analysis, the log K_{ow} value can also provide additional information about the potential of the substance to cause an effect in a particular bioassay [21].

There are many different fractionation techniques, but high-performance liquid chromatography (HPLC) is applied most frequently in EDA [10]. HPLC enables both on-line and off-line coupling to the bioassay. Off-line coupling is much more common, because frequently the time frame of the bioassay does not coincide with that of the fractionation [10]. HPLC as a fraction method in EDA was for example already used for the investigation of antiandrogenic effects in surface water [22] and endocrine effects in hospital wastewater [23]. Generally, organic solvents and additives used for the fractionation are not compatible with the following bioassay and therefore have to be eliminated before the bioassay is carried out. This can be achieved by carefully evaporating the solvent, which however may result in the loss of volatile compounds [24].

Only a few EDA applications with preparative gas chromatography (GC) are described as far [25]. GC is very suitable for volatile compounds due to its separation efficiency and the sensitive detection in combination with mass spectrometry. There are also huge data libraries for easier identification of effective compounds [24]. One application example is the separation of polycyclic aromatic hydrocarbons (PAH) like benzo[a]pyrene and investigation with the DR-LUC-bioassay for measurement of the potency to activate the dioxin receptor [26].

High-performance thin-layer chromatography (HPTLC) also has proven to be very suitable as fractionation method in EDA. It enables to perform the bioassay directly on the HPTLC plate and was applied most frequently to plant extracts [27] and foodstuffs [28,29] but also to environmental samples [30]. Different types of beer were examined and compared with regard to their estrogenic effects [28]. Bioassay batteries that investigated different endpoints were used for sunflower leaves [31], *Salvia*

miltiorrhize (sage) [32] or green tea [33]. One study investigated numerous cosmetic products, such as facial creams or sun creams, with a bioassay battery for their antioxidant potential, baseline toxicity, estrogenic and antibiotic effects [8]. Moreover HPTLC allows changes in the effects to be considered over time and over the application of a process, e.g. water treatment [34].

HPTLC as fractionation technique in EDA generally is based on an isocratic separation [8,32], that can be carried out quickly and with simple means and it can be easily adapted to new separation problems. With isocratic HPTLC, however, only peak capacities of 10 up to 35 can be achieved [35]. To minimize zone broadening and to increase peak capacity, the automated multiple development (AMD) is used in HPTLC. However, this gradient development is at the expense of the required time, which increases depending on the number of AMD separation steps applied [36].

Each separation technique (HPLC, GC or HPTLC) has different advantages and disadvantages for application in EDA. When using HPLC, a large number of different stationary phases are available for different fields of application and a broad substance spectrum can be captured. However, the selection of solvents for the mobile phase is limited and a solvent change has to be carried out before application of the bioassay. Gas chromatography can only be used for a limited amount of substances. However, it provides excellent peak capacities for fractionation and very well-developed databases for spectrum interpretation. In HPTLC, a variety of solvents can be used for separation, such that a broad substances spectrum can be captured. Furthermore, the HPTLC plate is solvent-free after separation and can be used directly for the following bioassay, but disadvantageously the peak capacity is limited.

4.2.2.1 Two-dimensional fractionation

In EDA, the fractionation method is used to reduce the number of candidates possibly causing the effect for easier identification of the true effective compounds. In the case of environmental samples such as surface or sewage water, however, numerous substances are still present in an effective fraction even after fractionation. So, identification of the effective substances remains time-consuming and ambitious and in some cases does not succeed. For this reason, two-dimensional techniques are used in EDA with the aim of increasing peak capacity and thus achieving a sufficient reduction of complexity [20].

Several two-dimensional fractionation methods are already in use for EDA. For example, there is a two-dimensional HPLC x HPLC method that uses the acetylcholinesterase inhibition assay (AChE assay) for the investigation of water samples [20]. One advantage of this application is the automatically performance. Both the 2D LC separation and the subsequent fractionation into a microtiter plate are performed completely automatically. After fractionation, the remaining solvent is evaporated from the microtiter plate and the bioassay is applied. By means of extremely small fractionation steps, up to 384 fractions can be separated with this application and examined for their effects. In addition, there are also applications of comprehensive HPTLC x HPTLC separations in EDA [37]. Therefore, the HPTLC plate is rotated 90° after the 1D and then the plate is separated again with another mobile phase. In the study, the pYES for the detection of estrogenic effects was performed after the second separation dimension. Moreover heart-cut combination of HPTLC + HPLC through extraction of zones from the HPTLC plate is also possible [38]. Therefore, not the entire sample is transferred to the

2D, but only a selected zone. While the bioassay for comprehensive separations is applied once after the 2D, the bioassay for the heart-cut application is applied only after the 1D.

Another possibility of 2D HPTLC was presented in this work. After the separation in the 1D, the bioassay was used to select the effective zones. These were extracted with the TLC-MS Interface and transferred to a second HPTLC plate. After the second separation, the bioassay was performed again for the detection of effects in the 2D (**Publication 1**).

4.2.2.2 Fraction collection for biotesting

The collection of fractions for the bioassay differs, depending on the used fractionation method (Figure 2). In column chromatography, the fractions are usually collected with an automatic fraction collector, depending on a certain time frame or on chromatographic peak detection [10]. By using HPTLC, the collection of fractions is not necessary, because of the direct application of the bioassay on the plate (in-plate fractionation) [24]. However, if the bioassay cannot be performed directly on the HPTLC plate, the TLC-MS Interface may be used as fraction collector. When using the Interface, an elution head is pressed onto the zone of the HPTLC plate to be extracted and an eluent solves the compounds of the zone from the plate [39]. The zones can be selected according to their retardation factors, or the entire retardation area may be divided in small sections that can be extracted at greater expense. Extracted zones can be examined with bioassays in the microtiter plate (**Publication 2**) or they may be applied to the HPTLC plate and separated again (**Publication 1**). Then the bioassay is performed after a 2D HPTLC separation. In addition, fractions obtained after column chromatography with the fraction collector could also be applied to the HPTLC plate. The bioassay can then be performed on the HPTLC plate, detecting also two-dimensional separated fractions.

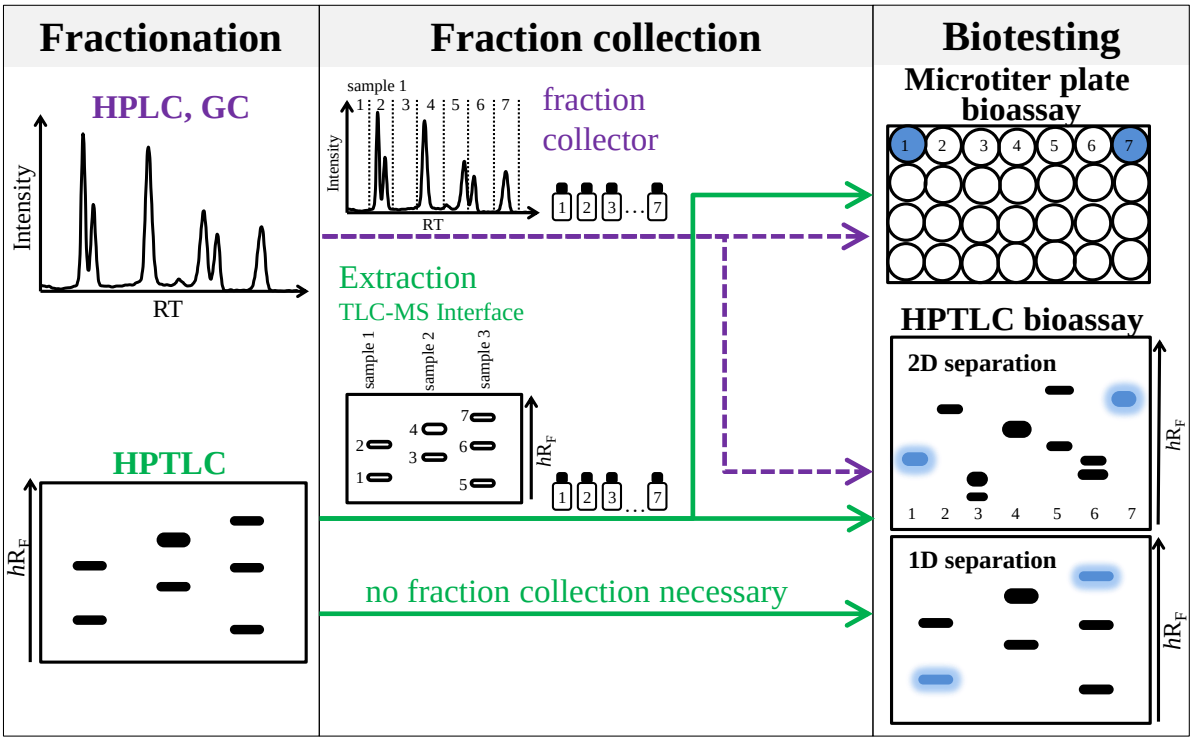


Figure 2: Possibilities for combination of HPLC, GC and HPTLC as fractionation methods with biotesting in the microtiter plate or on the HPTLC plate.

4.2.3 Biotesting

The biotesting is the key element of EDA, because it is used to determine the effects of the sample in a concentration dependent manner [10] and it gives biological relevance to the results [24]. The success of the EDA depends on the selection of suitable bioassays, which on the one hand must be relevant for the risk assessment and on the other hand should also be compatible with the samples to be examined. The selection of suitable bioassays shall be based on the following criteria [40]:

- **Required sample volume:** The volume required to perform the bioassay must be within a range that makes it possible to examine enriched sample extracts. The extracts are usually available with a volume of approximately 1 - 10 mL.
- **Tolerance to solvent residuals:** Due to the enrichment and separation of the samples, solvent residues must be expected, which must not negatively influence the bioassay.
- **Acceptable time exposure:** The separation into several fractions increases the effort for the analysis of a sample. Thus, the bioassay must be within an acceptable time frame for this analysis.
- **Sufficient sensitivity**: The bioassay has to be sufficiently sensitive to detect relevant substances. Due to the low concentrations of organic trace substances in samples, enrichment generally is required for biotesting.

Bioassays can be performed at the molecular, cellular (*in-vitro*) or whole organism (*in-vivo*) level. Various biotesting systems are available, whereby *in-vivo* assays are still used as standard in toxicology, because of their high relevance and their predictive power for (eco)toxicological effects. Through the use of intact organisms, both toxicokinetic and toxicodynamic aspects are taken into account with *in-vivo* bioassays [40]. However, *in-vitro* bioassays allow a higher throughput and thus the investigation of a larger sample number with lower costs compared to *in-vivo* bioassays. In addition, ethical aspects for avoiding experiments on living organisms speak against the widespread use of *in-vivo* bioassays (Figure 3).

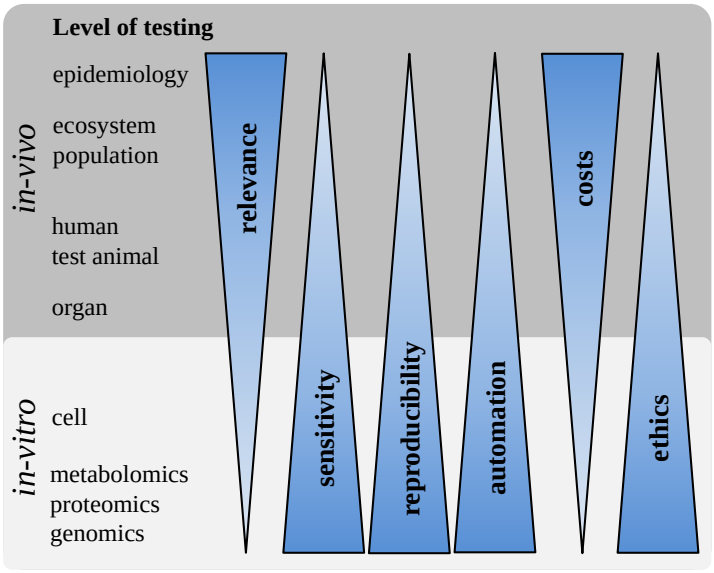


Figure 3: Comparison of toxicological testing with *in-vivo* and *in-vitro* bioassays [3].

So far, the transferability of effects, measured with an *in-vitro* bioassay, to complex systems such as mammals is still a major challenge. There are approaches, like the Adverse Outcome Pathway (AOP) concept, that shall facilitate the translation of mechanistic data into meaningful outcomes for environmental monitoring [41,42]. A bioassay that is used for environmental monitoring should meet two criteria: it has to be sensitive enough to detect chemicals of environmental concern and the measured results should be related to relevant responses in organisms. So, the AOP concept can also be used to select appropriate bioassays for EDA [43]. Cell-based bioassays cannot replace regulatory *in-vivo* bioassays, but they can provide additional information for risk assessment and substance prioritization [43]. They are extremely divers in their range of endpoints (Figure 4) and some of them have standardized protocols, such as the ISO (International Organization for Standardization) or DIN (German Institute for Standardization) guidelines.

4.2.3.1 Endpoints for investigation of aquatic samples

There are many different effects in the aquatic environment, so a single bioassay is not sufficient for the risk assessment of such a complex sample. Therefore, generally a bioassay battery (more than 3 different bioassays) is applied in EDA [3]. The battery should be designed in such a way that it can detect the effects usually occurring in environmental samples (Figure 4), including specific and non-specific endpoints [44]. In addition, bioassay batteries must be applicable in routine, which is why the selection of applied bioassays is focused on the most important environmental effects [12]. These include the induction of the xenobiotic metabolism, endocrine disruption and adaptive stress response [43]. Other important endpoints for the assessment of water quality are genotoxicity and neurotoxicity [45]. An overview of important effects in environmental analysis is shown in Figure 4.

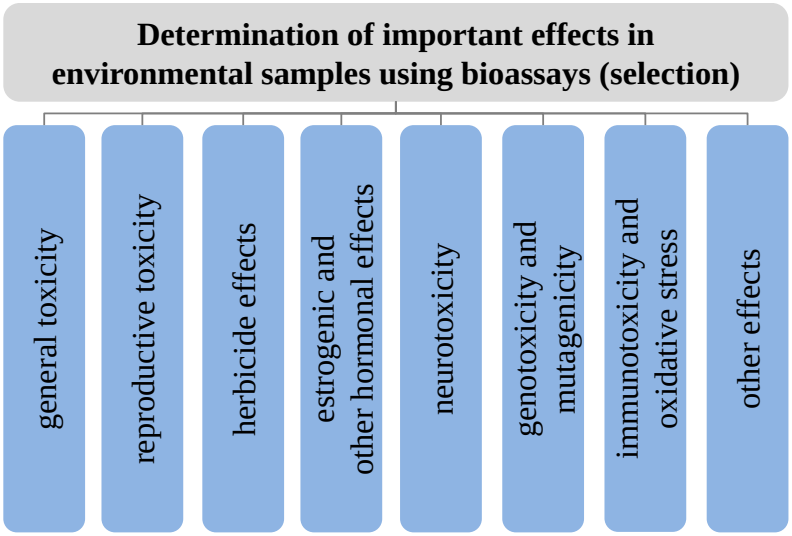


Figure 4: Overview of important effects in environmental analysis that can be recorded with bioassays [46].

Taking into account the reasons given above, to cover a broad effect spectrum with relevant endpoints and also to select bioassays that can be combined with the used enrichment and fractionation methods, the following five endpoints, each gathered with an *in-vitro* bioassay, were applied in the present work:

Genotoxicity

A substantial endpoint for monitoring of aquatic samples, which is also crucial for determination of limit values, is the genotoxicity [47]. Substances with a demonstrated genotoxic effect are allocated in drinking water to levels of ≤ 0.1 µg/L according to the health-related indicator value concept of Federal Environmental Agency (FEA) in Germany [48]. Genotoxic substances with a human relevant metabolism even get health-related indicator values of 0.01 µg/L in drinking water. Different *in-vitro* bioassays are available for the detection of genotoxic effects, such as the Ames assay, the micronucleus assay or the umu assay. The Ames assay with *Salmonella typhimurium* tester strains TA98 and TA100 with and without exogenous metabolic activation, is applied most frequently in water analysis [49]. The genetically modified strains carry mutations, which prevent the bacteria to grow without the addition of histidine to the growth media. Only bacteria that come in contact to mutagenic substances and therefore mutate back can grow on a histidine deficiency culture medium. The number of mutant colonies is counted and determines the mutagenic effect of the sample. In the micronucleus assay, occurrence of mutagenic substances is measured through appearance of micronuclei in Chinese ovary hamster (CHO) cells [50]. Micronuclei are small membrane bound DNA fragments that occur through deficient cell division. The cells are visually scored for the presence of micronuclei. Another bioassay that is frequently used is the umu assay with genetically modified *Salmonella typhimurium* TA1535/pSK1002 cells [51]. Presence of genotoxic substances leads to DNA damage, which triggers the expression of the *umuC*-gene, a part of the SOS-repair system. However, the *umuC*-gene is fused with the *lacZ*-reporter gene coding for the enzyme ß-galactosidase. Synthesis of ß-galactosidase is quantified through colorimetric detection of the substrate o-nitrophenyl-β-D-galactopyranoside (ONPG), which is cleaved through ß-galactosidase to the yellow product o-nitrophenol.

To date, no bioassay for the detection of genotoxic effects with metabolic activation can be performed directly on the HPTLC plate. One assay is available, which uses genetically modified yeasts for the detection of genotoxicity on the HPTLC plate [52]. However, only direct genotoxic effects can be captured, because the exogenous metabolic activation system does not work on the HPTLC plate so far. Since many substances exert an indirect genotoxic effect, in this work the umu assay in the microtiter plate was used as an alternative, which also enables metabolic activation (**Publication 2**).

Neurotoxicity

An important endpoint with a human-relevant mode of action is the neurotoxicity [53]. Substances with a proven neurotoxic effect therefore get a health-related indicator value of ≤ 0.3 µg/L in drinking water [48]. The detection of neurotoxic effects is difficult and very time-consuming. As very long observation periods are necessary for the detection of neurotoxic effects in the whole organism and the frequency of results is usually too low, more and more *in-vitro* bioassays are being used [54]. In EDA for example, the viability of primary cultured cerebellar granule neurons after incubation with fractions of environmental samples was investigated and neurotoxic brominated flame retardants could be identified [55]. For investigation of water samples, the *in-vitro* AChE inhibition assay is used most frequently [54]. This enzyme-based bioassay indicates the inhibition of the enzyme acetylcholinesterase that plays an important role in the transmission of stimuli in the synaptic cleft of the nerve cell. By using this assay, also unspecific binding of substances to the purified enzyme must be considered,

such that measured potential neurotoxic effects have to be confirmed through application of further bioassays [20].

After the development of the AChE inhibition assay in the microtiter plate in 1961 [56], the assay has also been performed on the HPTLC plate in 1964 [57] and has been widely used for investigation of potentially neurotoxic effects [27,58] (**Publication 1**). Particular emphasis in investigations was put on the carbamates and organophosphates [59], which specifically bind to the active center of the AChE [60]. These substance groups are often found in pesticides [61], but they are also used as drugs for the treatment of Alzheimer's disease [62,63] or Myasthenia gravis [64].

Estrogenic activity

Another important endpoint is the hormonal effect of substances and in particular their estrogenic activity [65]. In the case of estrogens, the effect already occurs in very low substance concentrations. For this reason, substances with proven estrogenic effects get a health-related indicator value of ≤ 0.01 µg/L in drinking water [48]. Three *in-vitro* bioassays are proposed as biotest battery for the detection of estrogenic effects in the health-related indicator value concept. In the first level of the battery, a reporter gene mediated test, such as the estrogen receptor-chemically activated luciferase expression® assay (ER-CALUX®) [66] or the Yeast estrogen screen (YES) [67] is to be applied. Additionally the H295R steroid genesis assay [68] is proposed for application. If estrogenic effects are detected with a reporter gene assay, an *in-vivo* reproduction assay, e.g. the *Potamopyrgus antipodarum* assay (New Zealand mud snail) [69], shall be performed in a subsequent step.

The YES, based on genetically modified *Saccharomyces cerevisiae* BJ3505 yeast cells is often used for the detection of estrogenic effects in water samples. The yeast cells contain a plasmid with the human ER-α. If estrogenic substances bind to the ER-α, a signalling cascade starts at the end of which the enzyme ß-galactosidase is formed. By adding a substrate that is cleaved by the ß-galactosidase, the estrogenic effect can be detected [67].

In 2004, the YES was performed on the HPTLC plate for the first time and named with the abbreviation pYES for planar YES [70]. Since then, several research groups have been working on the advancement of the bioassay on the HPTLC plate. This enabled the duration of the assay and the sharpness of the effective zones to be significantly optimized [71]. Several procedures for carrying out the assay were developed using different HPTLC plates (normal phase, NP and reversed phase, RP) and substrates (4-methylumbelliferyl-ß-D-galactopyranoside, MUG and resorufin-β-D-galactopyranoside) [72]. In some procedures the yeasts are sprayed onto the plate, while others immerse the plate in the yeast suspension [73,74].

Cytotoxicity

Cytotoxicity refers to the ability of substances to damage cells or tissues, which can cause cell death. Cytotoxic substances can also damage the microorganisms used in bioassays, so that their results cannot be interpreted. Therefore, the detection of cytotoxic effects as performed with the neutral red assay [75] or the MTT assay is very useful [76]. The MTT assay detects the metabolic activity of cells by reducing the yellow 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to violet MTT-formazan [77]. This reaction requires the reduction equivalents NADH and NADPH,

which are formed in the cells during glycolysis. If cytotoxic substances are present in a sample, they inhibit the glycolysis rate of the cells and as a result cause a lower conversion of the substrate MTT to MTT-formazan [76]. The MTT assay can be performed with different bacterial cells. For *Bacillus subtilis* (BS), for example, a commercial test kit is available.

In 1986, the *Bacillus subtilis* assay for the detection of cytotoxic effects was transferred to the HPTLC plate [78]. Since then, the assay has been continuously further developed [79,80] and was applied, for example, for the detection of antibacterial plant compounds [81] or antibiotics in milk [82].

Baseline toxicity

Baseline toxicity is used in ecotoxicology to determine general toxic effects. The bacterium *Aliivibrio fischeri* (AF), which is capable of bioluminescence, is frequently used for this purpose. The ability of the bacterium to bioluminescence is directly linked to the respiration via the electron transport chain, so that the influence of toxic substances on the cellular metabolic state is immediately reflected in the bioluminescence intensity [83]. The AF inhibition assay is used in a wide variety of applications, for example, to investigate the toxicity of organic and inorganic substances and metals, but also for liquid and solid samples. There is an application for the investigation of solid sediment particles with regard to their baseline toxicity. Depending on the incubation period, both acute and chronic effects on *Aliivibrio fischeri* can also be investigated [84]. Since 1978, the test has also been commercially available under the name Microtox®. In the meantime, there are several other suppliers who distribute the test (LUMIStox®, ToxAlert® or BioTox™). Since 1991, the bioassay has also been standardized by DIN [85].

The AF inhibition assay was first applied on the HPTLC plate in 1996 [86]. The AF inhibition assay with HPTLC is quick and easy to perform and therefore has already been used in several applications. For example, the assay is often used as initial assay in bioassay batteries [28,87]. It has also been used to investigate sunscreens [88], secondary metabolites in marine sponges [89], waste and landfill water [90] and marine sediments [91].

The following Figure 5 gives an overview of the bioassays used in this work:

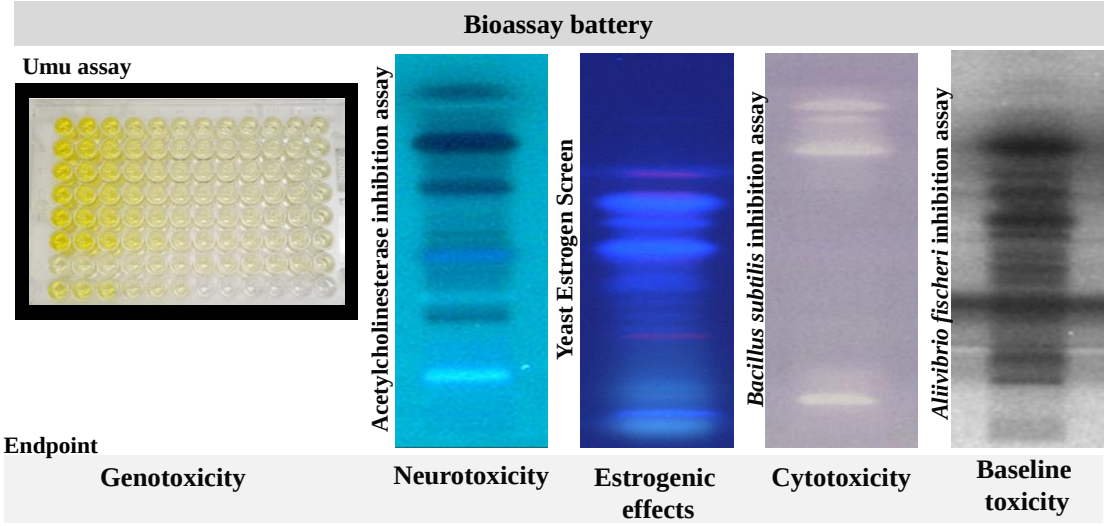


Figure 5: Overview of the bioassay battery used in this work.

4.2.3.2 Application of bioassay batteries for investigation of aquatic samples

Bioassay batteries have already been widely used for the analysis of aquatic environmental samples. One study investigated the behavior of effects during various water treatment processes such as ozonation, chlorination, UV-treatment or combinations thereof [13]. Thirty-six different bioassays were applied, covering a total of 18 different endpoints of effects. The nine investigated water samples showed correlations between the reduction of effects, the applied treatment steps and the respective endpoint investigated. For example, ozonation could trigger a significant decrease in oxidative stress, whereas UV-treatment led to a reduction in glucocorticoid activity. Chlorination reduced the genotoxicity and herbicidal activity of the tested samples. Groundwater infiltration in turn reduced the activity of all investigated endpoints except genotoxicity. In some cases, bioactivity increased as a result of a treatment step, suggesting the emergence of effective transformation products.

Effect behaviour during drinking water treatment was investigated in this thesis as part of the "WBA-BeReit"-project. 10 raw and drinking water samples of 7 water suppliers in Germany were investigated with the bioassay battery described in Figure 5. In contrast to the investigation named above [13], which was performed without fractionation of samples, in this approach EDA with HPTLC as fractionation method was applied. None of the water samples caused a potential genotoxic effect in the umu assay, but multiple effects were observed with the four bioassays on the HPTLC plate (Figure 6).

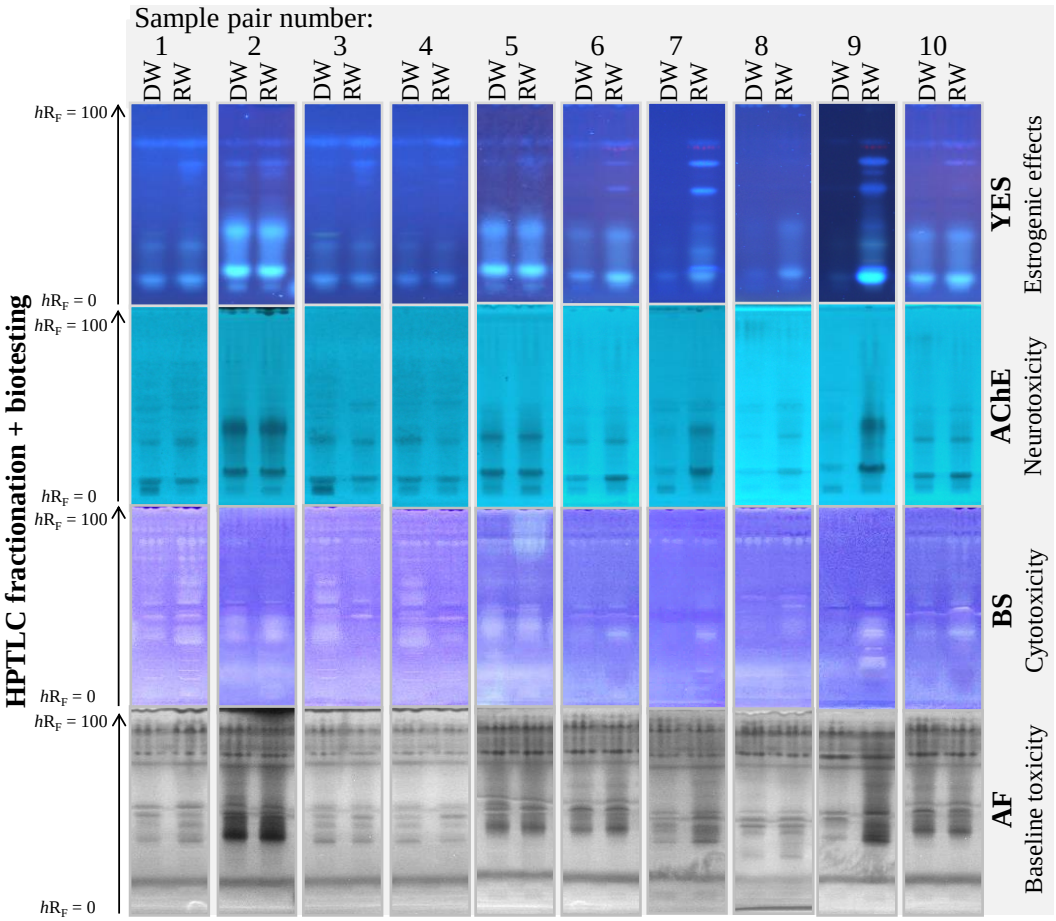
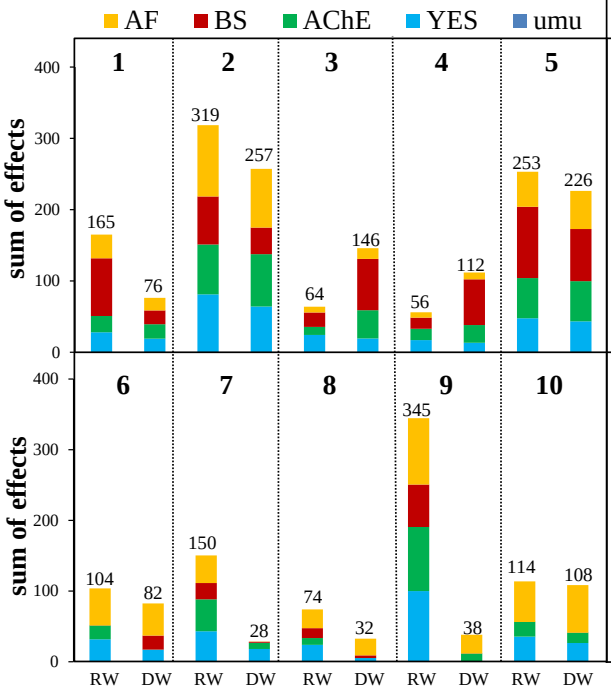


Figure 6: EDA with four endpoints of raw water (RW) and drinking water (DW) of different water suppliers from Germany; determined in the context of the project "WBA-BeReit", which was worked on in this thesis. Number 1 - 5: groundwater as raw water, number 6 - 10: surface water as raw water, modified from [19].

The effect pattern of each endpoint was similar in different waters studied, suggesting that similar substances are responsible for the detected effects (Figure 6). A comparison of the sample pairs revealed large differences with regard to the change of effects due to the treatment used. For example, for sample pair no. 2 almost no change in effects was achieved by the applied treatment, whereas the effect was significantly reduced in sample pair no. 9.

In summary, the HPTLC separation of the water samples resulted in an effect pattern of each investigated bioassay. On the basis of such effect patterns, a detailed examination and comparison of samples can be carried out. For a clear and simple description of the drinking water treatment however, the effects of the fractions were additionally summed up and standardised for each of the five bioassays. Thus, the sum of effects of a sample could be described by five numerical values (Figure 7).



	Sample pair	Treatment steps
Groundwater as raw water	1	filtration
	2	filtration
	3	filtration
	4	filtration
	5	filtration, disinfection*
Surface water as raw water	6	filtration, flocculation, disinfection*
	7	filtration, flocculation, O_3, AC, disinfection*
	8	filtration, O_3, disinfection*
	9	filtration, O_3, AC, disinfection*
	10	filtration, flocculation, disinfection*

*UV-disinfection and/or addition of chlorine-containing processing agents

Figure 7: Sum of effects of 10 raw- and drinking water sample pairs and the applied treatment steps, modified from [19]. RW: raw water, DW: drinking water, O_3: ozonation, AC: activated carbon filtration.

When comparing the raw water samples in Figure 7, there are strong fluctuations in the sum effects from 56 to 345, with no clear differences between the raw water sources used. The situation is different for the drinking water samples, examined after water treatment processes. The drinking water samples obtained from groundwater showed only little effect reduction and in two of the drinking waters (sample pair no. 3 and 4) the effects even increased. However, in the drinking waters from surface waters the effects could be significantly reduced. When comparing the differences in the sum effects with the treatment steps, it becomes apparent that more effect reduction could be obtained, when several different treatment steps were applied. In the sample pairs with successful effect reduction (sample pair no. 7, 8 and 9), ozonation and/or activated carbon filtration were used in addition to the other treatment techniques filtration, flocculation and disinfection. Whether these

treatment steps are particularly beneficial for effect reduction has to be investigated in subsequent studies.

4.2.4 Chemical analysis

Chemical analysis is crucial for identification of effective substances in EDA. However, it is only successful, if individual toxic substances or combinations of them cause the effect (toxicity drivers). If the measured effect is caused by the mixture itself, identification of all the compounds involved is not very promising. Three general approaches for identification of effective substances are applied in EDA [40]:

- **Mass balance approach:** Quantitative chemical target analysis of candidate compounds that might be responsible for the effect. The mass balance approach is used most frequently in EDA. For this purpose, known effective candidates are selected and searched in the effective samples via target analysis [12,15]. This approach is especially suitable, when specific bioassays are applied, in which only few substances usually cause the effect (e.g. YES). These substances are named toxicity drivers. If a toxicity driver is present in the sample, its concentration is determined and the effect of this substance concentration is checked. Thus it can be examined, whether the effect of a fraction can be explained by the identified substance [10].
- **Virtual effect-directed analysis:** In virtual EDA, multivariate statistical tools are used to obtain correlations between effects and the occurrence of substances or groups of substances. This approach is mainly used when the toxicity drivers of an endpoint are not known. A large sample number (more than 10, the more the better) is required for a meaningful application of virtual EDA. Additionally, investigated samples should contain a similar substance spectrum, which differs in the concentration of the substances and thus in the effect intensities. If these conditions are met, virtual EDA is very promising [92,93]. Thus, virtual EDA is suitable for investigation of time series of samples with the same origin and the same anthropogenic influences, but with seasonal variations in substance concentrations and effects. However, it must be considered that the linear course of the concentration level of a substance cannot simply be correlated with the sigmoid course of the dose-response curve of that substance. Thus, even a large concentration change of a substance can cause only a small change of the effect and *vice versa*, making the application of virtual EDA more difficult.
- **Higher tier effect-directed analysis:** Non-target strategy for identification of effective compounds in one or few samples. The higher tier EDA approach is used to identify previously unknown toxicity drivers (**Publication 3**). Non-target screening (NTS) is used, when the detected effects cannot be explained by the known toxicity drivers in the mass balance approach. Higher tier EDA creates direct cause-effect relationship, but it is time-consuming and expensive. Therefore it is used, if identification with the other two approaches is not successful or they cannot be applied [40].

There are several analytical methods available to apply chemical analysis in EDA, but in most cases HPLC-MS with electrospray ionization (ESI) is used [94]. When using selected ion monitoring in target analysis of the mass balance approach, triple-quadrupole mass spectrometers are applied in EDA. For NTS, time-of-flight or orbitrap mass spectrometers are mainly used, as they provide

sufficiently high mass accuracy (< 5 ppm at m/z 200) and resolution (> 10 000) for substance identification [40]. NTS workflow for EDA is divided in two steps: data acquisition of the effective samples and fractions and data evaluation. During data acquisition, full scan MS data of protonated or deprotonated molecular ions (or adducts) are recorded. In addition, collision-induced fragmentation generates MS/MS data of the molecules, which can provide structural information. After data acquisition, the data evaluation is performed, which is important for reducing the quantity and complexity of the data. During the data evaluation the selection of the peaks (peak finding), the subtraction of blank signals as well as a componentization of the signals takes place, with which the individual signals (isotopologues, adducts, in-source fragments) are to be assigned to one compound. By measuring a sample several times, additional instrumental noise signals can be reduced. When measuring several samples, the signals are combined in a common list by an alignment. The elaborate data evaluation results in a list of valid signals that can be used for the subsequent identification strategy [95].

An important step in the identification of effective substances is the allocation of the exact masses in the list to molecular formulas. Therefore, mass accuracy and resolution of the mass spectrometer used are decisively, as are the isotope pattern and fragment information. The molecular formulas determined are then compared with possible structural formulas. Databases such as ChemSpider or Pubchem, which contain millions of substances, are helpful there. Databases on the toxicity of substances can also help to identify the effective substance. Due to the large number of different substances, many structural formulas can usually be assigned to one molecular formula, so that this must be checked by additional structural information, such as the MS/MS spectra. However, a clear level 1 identification of a substance can only be achieved by means of a reference standard [96]. Despite the reduction of sample complexity via fractionation, identification of (effective) substances still is very challenging. Possible reasons for failing identification attempts are very complex samples, such that still many different substances occur in one fraction or the lack of structure and spectra databases as well as the generally high manual effort for NTS [94].

4.2.4.1 Application of identification approaches in EDA

There are various applications of the three identification approaches named above, including combinations of them. Different surface and wastewaters in Europe were investigated for example for their estrogenic effects with five different *in-vitro* bioassays (ER-CALUX®, MELN, Hela-9903, GeneBLAzer, pYES). In parallel, three estrogens from the “watch-list” of the water framework directive (17ß-estradiol, estrone and 17α-ethinylestradiol) were investigated using the mass balance approach. Determined 17ß-estradiol equivalents of the five bioassays were comparable. The measured substance concentrations differed strongly between sampling sites and from wastewater to surface water. However, there was a strong correlation between the results of the bioassays and those of the chemical analysis [65].

Also in this thesis, within the scope of the “WBA-BeReit”-research project, a chemical analysis using the mass balance approach was carried out. Twenty raw and drinking water samples from different water suppliers were analysed with the pYES for their estrogenic effects and also with HPLC-MS/MS analysis for five estrogens (17ß-estradiol, estrone, estriol, 17α-ethinylestradiol and 17α-estradiol). Three estrogens (17ß-estradiol, estrone and estriol) were detected and quantified in different raw

waters. Through performing the pYES with the quantified concentration of the detected estrogens, the measured estrogenic effect in the respective zones could be fully explained [19].

Compared to the mass balance approach, there are fewer applications in the area of virtual EDA. One study described the use of virtual EDA to identify antiandrogenic effects in soil samples of a chemical park. Therefore, an approach was developed in which first a virtual fractionation of the samples and then a virtual quantitative structure-retention relationship was established. Antiandrogen effects were detected in 8 out of 18 soil samples with the AR-antagonist assay. Multivariate statistical methods were used to select 76 peaks that could cause the effects. By comparison with the classical EDA results, 90 % of those peaks could also be confirmed in effective fractions of the soil samples. Thus identification of key toxicants in samples should become more efficient and faster with virtual strategies [97].

In 2018, the higher tier EDA approach was used for the identification of antiandrogenic substances in surface water. The samples were fractionated using four different stationary HPLC phases and the fractions were examined for their effect using the anti-AR CALUX® assay. One effective fraction occurred and an NTS by HPLC-MS/MS analysis was applied. The highly potent antiandrogenic substance 4-methyl-7-diethylaminocoumarin and two derivatives thereof were identified. The detected effect in the surface water could be explained almost completely by quantifying the identified substances. Moreover, the antiandrogen effect of the substances was confirmed by further *in-vivo* bioassays [22].

The higher tier EDA approach was also applied in this thesis for identification of effective compounds in the project “WBA-BeReit”. The *Bacillus subtilis* inhibition assay was used to detect cytotoxic effects in different raw and drinking waters. One effective zone occurred simultaneously in several of the examined raw waters and it was thus suspected that the detected effect was caused by the same toxicant. Therefore, the effective zones were extracted and an NTS using HPLC-HRMS/MS was performed. In the extracted zones, a total of 10 substances could be clearly identified by means of reference standards, whereby these were exclusively drugs and their transformation products. Only two of the ten substances showed a cytotoxic effect in the BS inhibition assay, namely clindamycin-sulfoxide and candesartan. While candesartan is widely used as an antihypertensive, clindamycin-sulfoxide is a transformation product of clindamycin, which is used in many ways as an antibiotic. However, the cytotoxic effect of the extracted zone could not be explained by these substances, which only exert a cytotoxic effect in higher concentrations than those contained in the samples. Therefore, further unknown substances must occur in the extracted zone, which together with candesartan and clindamycin-sulfoxide trigger the cytotoxic effect [19].

4.2.4.2 Examination of water treatment processes with chemical analysis and effect detection

Additionally to the identification of effective substances, chemical analysis with NTS is also be used to describe variability of substance concentrations during water treatment processes [98]. In this non-target process evaluation, the signals are divided into five categories according to the ratio of their signal intensity (fold change, fc) from drinking water (DW) to raw water (RW):

$$fc = \frac{signal\ intensity_{DW}}{signal\ intensity_{RW}}$$

- Elimination (**dark green**): fc < 0,2
- Decrease (**green**): 0,2 ≤ fc < 0,5
- Consistency (**grey**): 0,5 ≤ fc ≤ 2,0
- Increase (**yellow**): 2,0 < fc ≤ 5,0
- Formation (**red**): 5,0 < fc

NT process evaluation can be used to correlate changes in substance concentrations with changes in effects, even without primarily identifying effective substances. This was applied in this thesis for the examined raw and drinking waters of different water suppliers. The raw and drinking water samples showed a similar behaviour in the results of NT process evaluation and effect process evaluation. If the effects decreased significantly through applied treatment steps, as for example in sample pair no. 7, this was also reflected in the NT process evaluation with a large number of signal ratios in the category "Elimination" or "Decrease" (Figure 8). In the case of sample pairs with only little effect reduction from raw water to drinking water, a large number of signals was detected in the category "Consistency".

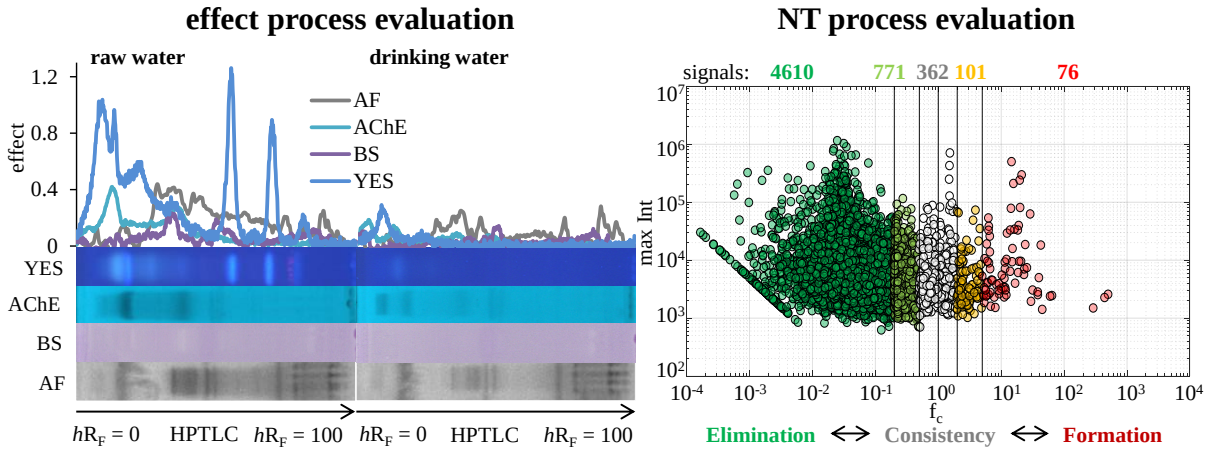


Figure 8: Comparison of the effect process evaluation with the NT process evaluation of sample pair no. 7 (Figure 7).

The ratio of the sum effect of drinking water to raw water is a measure to describe the effectiveness of the treatment with regard to the removal of the effects. In the same way, the ratio of the number of detectable signals in drinking water and raw water in the NT process evaluation can describe the effectiveness of the treatment with regard to the removal of substances. A comparison of these two measures shows that treatment with ozonation and/or activated carbon filtration in raw water tends to best reduce the measured effects and NT signals.

5 Discussion

5.1 Organic trace substances in the aquatic environment

In the present work, EDA with HPTLC was applied for the analysis of effective trace compounds in the aquatic environment. Organic trace substances generally occur in the environment in low concentrations (up to µg/L), such that the working range of EDA has to be considered (Figure 9). While chemical analysis via LC-MS/(MS) can still cover the ng/L-range without enrichment, the working range of the bioassays used in this thesis spans from µg/L to mg/L (exception YES: ng/L - µg/L). Therefore, without sample enrichment, the usual environmental concentrations would not be detectable with the bioassays, so in this thesis, environmental samples were enriched by SPE with an enrichment factor of 1000.

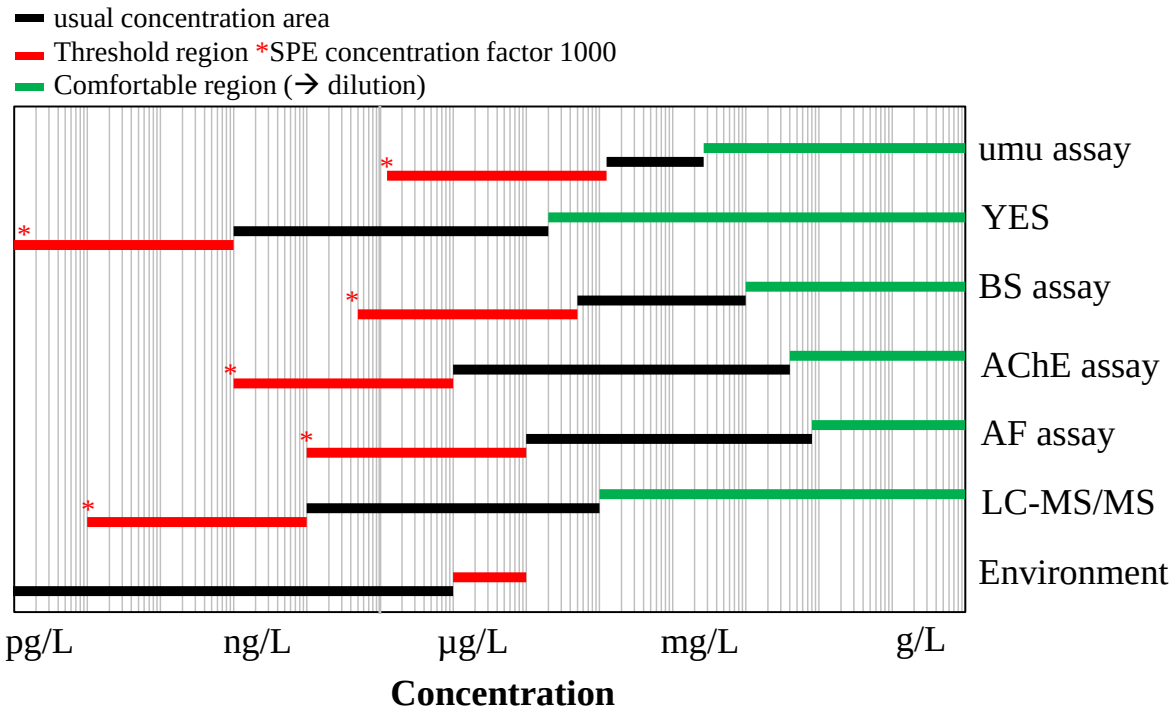


Figure 9: Concentration range of environmental organic trace substances in comparison with the working range of LC-MS/(MS) and the applied bioassays on the HPTLC plate (exception umu assay).

There are large differences in the sensitivity of the bioassays used in this work. While the YES can detect substances down to the ng/L-range without enrichment, with the umu assay the genotoxic effects can only be gathered in much higher substance concentrations. Thus, the umu assay is not able to detect low environmental concentrations of trace compounds even with a 1000-fold enrichment and therefore is only suitable to limited extent for the use in water quality assessment. Rather, the umu assay is suitable for regulatory investigations, where higher substance concentrations are available instead of the often low environmental concentrations. In that way, the umu assay is also used in the health-related indicator value concept for drinking water of the FEA in Germany. But for water quality assessment and drinking water monitoring, there is a need for more sensitive bioassays for genotoxicity detection. A switch to human reporter-gene cell lines, such as the p53-CALUX® assay

[99] may likely lead to an increase of sensitivity, so that the endpoint genotoxicity can successfully gather the effects of substances contained in water in low concentrations.

5.2 Effect-directed analysis

Sample preparation

SPE is the most widely used enrichment method for application in EDA, because it is suitable for enrichment of substances in a wide polarity range. But small and polar substances in particular, can only be enriched to a limited extent by SPE [18]. In the research project "WBA-BeReit", good recoveries for polar and ionic substances were achieved with vacuum concentration and freeze drying, but they also cause enrichment of salt content, which interfered with the HPTLC separation. Moreover, only enrichment factors of 50 - 100 could be achieved with these methods, so that substance concentrations did not fell within the working range of EDA [19]. In order to be able to record polar substances with EDA, further investigations for the enrichment of these substances are required in the future. For example, multi-layer SPE materials may be used, which at the same time enable an enrichment of more polar substances and also achieve higher enrichment factors required for EDA [18].

Fractionation

EDA with HPTLC as fractionation method provides the advantage that effects can be detected quickly and due to the possibility of applying up to 15 samples simultaneously on the HPTLC plate, a high sample throughput is achieved. However, the disadvantage of HPTLC is the limited peak capacity in comparison to HPLC and GC. For this reason, a selective 2D EDA method was developed in this work (**Publication 1**). By using a heart-cut approach, only effective zones were transferred from 1D to the 2D and separated with another mobile phase, before the bioassay was applied again.

The developed selective 2D EDA introduced a new method for the more selective detection of potential neurotoxic effects. There are already applications of comprehensive 2D HPTLC on a HPTLC plate with following biotesting [37], but such approaches carry out first the two separations before the bioassay can be performed. Thus, it cannot be checked whether effects occur in the bioassay at all and whether a separation in the second dimension would be necessary. There are also applications where the effective zones are extracted from the plate after separation in the 1D and separated in the 2D by HPLC [38]. However, the bioassay can only be used after the 1D, so that no further prioritization of the effective ingredients is possible after the second separation.

To increase the peak capacity by 2D chromatography, orthogonal separation techniques are necessary. In order to obtain orthogonal separation mechanisms, it would be useful to change the selectivity of the stationary phase. However, in EDA with HPTLC, it is crucial that stationary phase parameters (pH value, wettability of the HPTLC plate, etc.) are compatible with the applied bioassay. Therefore, the same stationary phase, but different mobile phases for 2D chromatography were used in this thesis.

2D HPTLC enabled peak capacities of up to 208 in this work (**Publication 1**). Compared to 1D HPTLC gradient development, an increase of peak capacity by a factor of about 7 was reached. In comparison, 1D HPLC provides peak capacities of approximately 100 - 1500, while 2D HPLC can

achieve peak capacities of several thousand [100,101]. So, with 2D HPTLC only peak capacities in the lower 1D HPLC range can be achieved. In this work, however, the application of 2D EDA for surface water and sewage water showed that a peak capacity of around 200 is sufficient for the prioritization so that only a few signals remained as possible effective candidates.

Biotesting

One advantage of EDA with HPTLC is that the bioassay can be performed directly on the HPTLC plate, allowing a pattern comparison of the effects of different bioassays. However, the transfer of bioassays to the HPTLC plate considers complex method adjustments and validation steps [72,80] and not each bioassay can be successfully transferred. For example, the umu assay with metabolic activation cannot be performed on the HPTLC plate yet, because the enzymes of xenobiotic metabolism are not stable on the plate [102]. To bypass direct application of the xenobiotic enzymes to the plate, the metabolic activation can be performed in solution and the metabolized sample is applied to the HPTLC plate for separation. Another option, which was applied in this thesis, is to investigate via HPTLC separated fractions with the umu assay in the microtiter plate (**Publication 2**).

The developed procedure for linking the umu assay with EDA and HPTLC enabled the assignment of a transformation product of the substance metformin to a genotoxic effect. By repeated extraction of zones over the entire retardation area and investigation of these fractions with the umu assay, it was possible to prioritize to one fraction with a genotoxic effect. Through chemical analysis via HPLC-MS/MS, the effective transformation product could be clearly identified. So, in this work a possibility was developed to successfully carry out the EDA with HPTLC even without a direct application of the bioassay to the plate. This method may be expanded to further bioassays with sensitive or adherent cells that cannot be applied directly on the plate. As an alternative, the fractionation method may be changed from HPTLC to HPLC. In this case, it would be possible to collect the fractions of the samples directly in the microtiter plate for the following bioassay [23]. However, the fractions have to be solved in a solvent that is compatible with the bioassay so that the eluent of the HPLC has to be evaporated. Additionally, to reach the working range of the bioassay, a large sample volume must be injected onto the HPLC column. But such high substance concentrations may cause capacity problems on the column. Alternatively, the HPLC separation may be repeated several times to increase the substance concentration in the fractions.

In spite of the effort for the transfer, it would be desirable to be able to apply as many different endpoints as possible on the HPTLC plate. Hence, the effect patterns of different samples may be easily compared within an endpoint and also between different endpoints of effects. There are already approaches to detect for example androgenic and dioxin-like effects directly on the HPTLC plate. Approaches for the assessment of further hormone-like effects such as glucocorticoids or progesterones have also already been published [103].

In this thesis, five bioassays were already applied as bioassay battery, so that a wide range of effects could be recorded. However, the applied bioassays differ in their relevance depending on the research question to be investigated. The AF assay and also the BS assay for the detection of baseline toxicity and cytotoxic effects, respectively, are very well suited for the detection of eco-toxicological effects in water samples. They can detect a broad substance spectrum and thus give a good overview of the

exposure of different samples. For monitoring of drinking water, on the other hand, human-toxic effects are of more relevance. These two assays (AF and BS assay) are of secondary importance, because the bioassay results cannot be transferred to higher organisms. This is different with the three other bioassays applied in this thesis. Due to the mechanisms of action, which can also take place in higher organisms, the transferability is rather given, albeit with limitations. For example, the AChE assay is susceptible to the denaturation of the free enzyme by matrix compounds in the samples, which would not occur if the enzyme was present in an intact cell. False-positive results caused by non-specific effects must be expected with this assay. For this reason, the AChE assay is to be understood as an initial assay, whose potential neurotoxic results must be confirmed by further and often more complex *in-vivo* bioassays.

In order to cover questions that assess effects with regard to more human-toxicological relevance, the bioassay battery used in this thesis should be supplemented by further bioassays. For this purpose, human cell lines may be used that contain the human metabolic cell equipment and so may achieve a greater acceptance of the EDA results.

Chemical analysis

In this thesis, a 2D EDA workflow for easier identification of effective substances was developed (**Publication 3**). A proof of concept with six spiked neurotoxic substances in different water samples showed that the workflow can be successfully applied and that the substances can be recovered after the prioritization steps. The efficiency of the workflow for identification of unknown substances could be demonstrated by identifying potentially neurotoxic substances.

However, the necessary repeated extraction of samples results in a loss of substance concentration during the workflow. One opportunity to limit this loss may be a flexible elution head that could be adapted to the size of the zone to be extracted. Moreover, the extraction step with the TLC-MS Interface still requires a great deal of manual effort and skills on the part of the user, as the zones have to be determined by using a coordinate system on the extractor. A great simplification would be the automation of the extraction steps via the TLC-MS Interface, so that effective zones could be automatically transferred from the 1D to the 2D and to the mass spectrometric measurement. Overall, the workflow is particularly well suited when unknown effects occur at high intensity, as is the case with sewage or surface water rather than drinking water.

If the identification of the effective substance is still not successful, different reasons may be responsible. These may be the lack of structural and spectral databases and of databases with toxicity information and the generally high manual effort for NTS may also lead to failed identification attempts.

5.3 Research project „WBA-BeReit"

In this thesis, the research project "WBA-BeReit" was performed, where EDA with HPTLC was used in a real world application for the investigation of raw and drinking water from different water suppliers in Germany. It should be investigated whether EDA with HPTLC is suitable for assessing the behavior of effects of organic trace substances in drinking water treatment. The results showed clear differences in the effects depending on the raw water and the treatment methods used, so that an initial evaluation of the various treatment steps could take place.

The description of the treatment process with EDA was carried out by summing up the effects over the entire retardation area. This allowed a simple and clear comparison of the examined samples, but the information obtained by the fractionation was lost through the summation. In further work, it would be advisable to introduce a retardation factor-dependent comparison of the effects over the treatment process. Then the investigated samples may be compared better, such that even small differences in the effects could be detected. Since so far, there is the risk, that small differences in the effects are masked by the summation of all effects.

The investigation of treatment processes during drinking water production carried out to date have shown that the effects tend to be most effectively removed by the use of the treatment processes ozonation and/or activated carbon filtration. However, so far only random samples of different water suppliers could be examined in the project, so that the results would have to be consolidated by further investigations. It would also be important for the evaluation to consider single treatment steps instead of the entire treatment process.

Finally, a comparison showed a strong correlation between EDA and NTS results with regard to the assessment of treatment processes. This relationship contradicts the assumption that only a few toxicity drivers are responsible for the detected effects. However, non-specific bioassays, such as the AF assay and BS assay, were also applied in this work, which enable the detection of the multitude of substances in a sample. Also the AChE assay reacts to a large number of substances and thus reflects the complexity of the samples. With the help of these bioassays, as with NTS, the large number of substances contained in the samples is recorded and thus explains the very good correlation between the results of EDA and NTS.

However, in comparison to NTS data, the EDA also provides a statement on the relevance of the substances. For example, a process with a lot of substance reduction could result in only one substance with a relevant effect that is decisive for the evaluation of the process. Thus, EDA not only provides a statement on the quantity of signal changes, but also assigns a relevance to the occurring substances and is, therefore, very suitable as a supplement to the NTS.

6 Conclusion

The detection of effects is of vital importance for water quality assessment, because only by detecting the effects, a possible arising risk of a sample can be estimated. However, if relevant effects occur in a sample, the identification of the bioactive substances is essential. Therefore, in this thesis a strategy for the prioritization of bioactive trace compounds with 2D EDA was developed. The aim of the strategy was to reduce the complexity of effective fractions so that they could be prioritized to only a few candidate signals and the effective substance(s) could be identified more easily. Since the substances responsible for the effect are known, targeted measures can be taken to reduce or even prevent their entry in the aquatic environment. Thus, EDA makes an important contribution to the water quality assessment and subsequently also for drinking water production.

The developed 2D EDA with HPTLC and HPLC-HRMS approach used the AChE assay for the detection of neurotoxic compounds in this thesis, but it could also be transferred to further endpoints in the future. A broad application of the strategy with different water samples could show additional aspects to optimize the identification of effective compounds.

The successful application of the EDA with HPTLC for the evaluation of processes during drinking water treatment could be demonstrated in the research project “WBA-BeReit”. By investigating raw and drinking water from various water suppliers, it was possible to describe the treatment process in terms of effect reduction, whereby ozonation and activated carbon filtration tended to be most effective in reducing the effect. This information gain through the process description with the EDA opens up new possibilities in the quality assurance of drinking water. However, in order to be able to make even more precise statements about the individual treatment processes, further investigations are necessary, which also look at individual treatment steps.

By connecting bioassays in the microtiter plate to EDA with HPTLC, the application range of the method could be considerably extended. This allows application of additional endpoints, such that monitoring of aquatic samples gets broader and the detectable effect spectrum can be expanded. In order to record more effects in the future that could also be relevant for humans, additional bioassays must be used to ensure better transferability of the results to higher organisms. In addition, there is a need for more sensitive bioassays for the water quality assessment that are able to detect also very low environmental concentrations of trace compounds.

7 References

[1] S.A. Snyder, F.D.L. Leusch, "In vitro bioassays: current status and future applications for water management", Global Water Research Coalition (2018).

[2] K. Fent, "Ökotoxikologie - Umweltchemie Toxikologie Ökologie", Georg Thieme Verlag, Stuttgart (2013).

[3] B.I. Escher, F.D.L. Leusch, "Bioanalytical tools in water quality assessment", IWA Publishing, London (2012).

[4] R.P. Schwarzenbach, B.I. Escher, K. Fenner, et al., "The challenge of micropollutants in aquatic systems" *Science* 313 (2006) p. 1072-1077.

[5] A.B.A. Boxall, C.J. Sinclair, K. Fenner, et al., "When synthetic chemicals degrade in the environment" *Environ. Sci. Technol.* 38 (2004) p. 368A-375A.

[6] W. Brack, "Effect-directed analysis: a promising tool for the identification of organic toxicants in complex mixtures?" *Anal. Bioanal. Chem.* 377 (2003) p. 397-407.

[7] S. Krüger, L. Hüsken, R. Fornasari, et al., "Effect-directed fingerprints of 77 botanical extracts via a generic high-performance thin-layer chromatography method combined with assays and mass spectrometry" *J. Chromatogr. A* 1529 (2017) p. 93-106.

[8] C. Stiefel, T. Schubert, G.E. Morlock, "Bioprofiling of cosmetics with focus on streamlined coumarin analysis" *ACS Omega* 2 (2017) p. 5242-5250.

[9] T. Chasset, T.T. Häbe, P. Ristivojevic, et al., "Profiling and classification of French propolis by combined multivariate data analysis of planar chromatograms and scanning direct analysis in real time mass spectra" *J. Chromatogr. A* 1465 (2016) p. 197-204.

[10] W. Brack, S. Aït-Aïssa, R.M. Burgess, et al., "Effect-directed analysis supporting monitoring of aquatic environments - An in-depth overview" *Sci. Total Environ.* 544 (2016) p. 1073-1118.

[11] P.A. Neale, W. Brack, S. Aït-Aïssa, et al., "Solid-phase extraction as sample preparation of water samples for cell-based and other in vitro bioassays" *Environ. Sci.: Processes Impacts* 20 (2018) p. 493-504.

[12] M. König, B.I. Escher, P.A. Neale, et al., "Impact of untreated wastewater on a major European river evaluated with a combination of in vitro bioassays and chemical analysis" *Environ. Pollut.* 220, Part B (2017) p. 1220-1230.

[13] A. Jia, B.I. Escher, F.D.L. Leusch, et al., "In vitro bioassays to evaluate complex chemical mixtures in recycled water" *Water Res.* 80 (2015) p. 1-11.

[14] F.D.L. Leusch, P.A. Neale, A. Hebert, et al., "Analysis of the sensitivity of in vitro bioassays for androgenic, progestagenic, glucocorticoid, thyroid and estrogenic activity: Suitability for drinking and environmental waters" *Environ. Int.* 99 (2017) p. 120-130.

[15] P.A. Neale, N.A. Munz, S. Aït-Aïssa, et al., "Integrating chemical analysis and bioanalysis to evaluate the contribution of wastewater effluent on the micropollutant burden in small streams" *Sci. Total Environ.* 576 (2017) p. 785-795.

[16] P.A. Neale, B.I. Escher, "Coextracted dissolved organic carbon has a suppressive effect on the acetylcholinesterase inhibition assay" *Environ. Toxicol. Chem.* 32 (2013) p. 1526-1534.

[17] T. Schulze, M. Ahel, J. Ahlheim, et al., "Assessment of a novel device for onsite integrative large-volume solid phase extraction of water samples to enable a comprehensive chemical and effect-based analysis" *Sci. Total Environ.* 581-582 (2017) p. 350-358.

[18] N. Köke, D. Zahn, T.P. Knepper, et al., "Multi-layer solid-phase extraction and evaporation - enrichment methods for polar organic chemicals from aqueous matrices" *Anal. Bioanal. Chem.* 410 (2018) p. 2403-2411.

[19] W. Schulz, O. Happel, L. Stütz, et al., "Wirkungsbezogene Analytik als neuer Ansatz zur orientierenden Bewertung organischer Spurenstoffe in Rohwasserressourcen zur Trinkwassergewinnung und bei Aufbereitungsprozessen (WBA-BeReit)", DVGW - Deutscher Verein des Gas- und Wasserfachs (2019) p. 144.

[20] X. Ouyang, P.E.G. Leonards, Z. Tousova, et al., "Rapid screening of acetylcholinesterase inhibitors by effect-directed analysis using LC × LC fractionation, a high throughput in vitro assay, and parallel identification by time of flight mass spectrometry" *Anal. Chem.* 88 (2016) p. 2353-2360.

[21] P. De Voogt, J.W.M. Wegener, J. Klamer, et al., "Prediction of environmental fate and effects of heteroatomic polycyclic aromatics by QSARs: The position of n-octanol/water partition coefficients" *Biomed. Environ. Sci.* 1 (1988) p. 194-209.

[22] M. Muschket, C. Di Paolo, A.J. Tindall, et al., "Identification of unknown antiandrogenic compounds in surface waters by effect-directed analysis (EDA) using a parallel fractionation approach" *Environ. Sci. Technol.* 52 (2018) p. 288-297.

[23] F. Itzel, K.S. Jewell, J. Leonhardt, et al., "Comprehensive analysis of antagonistic endocrine activity during ozone treatment of hospital wastewater" *Sci. Total Environ.* 624 (2018) p. 1443-1454.

[24] M.G. Weller, "A unifying review of bioassay-guided fractionation, effect-directed analysis and related techniques" *Sensors* 12 (2012) p. 9181-9209.

[25] C. Meinert, E. Schymanski, E. Küster, et al., "Application of preparative capillary gas chromatography (pcGC), automated structure generation and mutagenicity prediction to improve effect-directed analysis of genotoxicants in a contaminated groundwater" *Environ. Sci. Pollut. Res.* 17 (2010) p. 885-897.

[26] E. Pieke, F. Heus, J.H. Kamstra, et al., "High-resolution fractionation after gas chromatography for effect-directed analysis" *Anal. Chem.* 85 (2013) p. 8204-8211.

[27] Á.M. Móricz, P.G. Ott, T.T. Häbe, et al., "Effect-directed discovery of bioactive compounds followed by highly targeted characterization, isolation and identification, exemplarily shown for Solidago virgaurea" *Anal. Chem.* 88 (2016) p. 8202-8209.

[28] P.M. Ristivojević, G.E. Morlock, "Effect-directed classification of biological, biochemical and chemical profiles of 50 German beers" *Food Chem.* 260 (2018) p. 344-353.

[29] D. Schick, W. Schwack, "Detection of estrogen active compounds in hops by planar yeast estrogen screen" *J. Chromatogr. A* 1532 (2018) p. 191-197.

[30] I.M. Choma, E.M. Grzelak, "Bioautography detection in thin-layer chromatography" *J. Chromatogr. A* 1218 (2011) p. 2684-2691.

[31] Á.M. Móricz, P.G. Ott, I. Yüce, et al., "Effect-directed analysis via hyphenated high-performance thin-layer chromatography for bioanalytical profiling of sunflower leaves" *J. Chromatogr. A* 1533 (2018) p. 213-220.

[32] E. Azadniya, G.E. Morlock, "Bioprofiling of Salvia miltiorrhiza via planar chromatography linked to (bio)assays, high resolution mass spectrometry and nuclear magnetic resonance spectroscopy" *J. Chromatogr. A* 1533 (2018) p. 180-192.

[33] I.M. Choma, M. Olszowy, M. Studziński, et al., "Determination of chlorogenic acid, polyphenols and antioxidants in green coffee by thin-layer chromatography, effect-directed analysis and dot blot – comparison to HPLC and spectrophotometry methods" *J. Sep. Sci.* 42 (2019) p. 1542-1549.

[34] I. Klingelhöfer, G.E. Morlock, "Bioprofiling of surface/wastewater and bioquantitation of discovered endocrine-active compounds by streamlined direct bioautography" *Anal. Chem.* 87 (2015) p. 11098-11104.

[35] H.-P. Frey, K. Zieloff, "Qualitative und quantitative Dünnschichtchromatographie: Grundlagen und Praxis", VCH, Weinheim (1992).

[36] L. Membrado, V.L. Cebolla, C. Jarne, et al., in: Instrumental thin-layer chromatography, "Automated Multiple Development" Elsevier, Boston (2015) p. 73-105.

[37] Y.A. Witos, B. Milz, B. Spangenberg, "Two-dimensional thin-layer chromatography of 17α-ethinylestradiol on RP-18 W plate, detected by effect-directed analysis using the YES test" *J. Planar Chromatogr.--Mod. TLC* 30 (2017) p. 136-141.

[38] T. Mroczek, "Qualitative and quantitative two-dimensional thin-layer chromatography/high performance liquid chromatography/diode-array/electrospray-ionization-time-of-flight mass spectrometry of cholinesterase inhibitors" *J. Pharm. Biomed. Anal.* 129 (2016) p. 155-162.

[39] G. Morlock, W. Schwack, "Coupling of planar chromatography to mass spectrometry" *TrAC, Trends Anal. Chem.* 29 (2010) p. 1157-1171.

[40] W. Brack, "Effect-directed analysis of complex environmental contamination", Springer Berlin Heidelberg (2011).

[41] G.T. Ankley, R.S. Bennett, R.J. Erickson, et al., "Adverse outcome pathways: A conceptual framework to support ecotoxicology research and risk assessment" *Environ. Toxicol. Chem.* 29 (2010) p. 730-741.

[42] D.L. Villeneuve, D. Crump, N. Garcia-Reyero, et al., "Adverse Outcome Pathway (AOP) development I: Strategies and principles" *Toxicol. Sci.* 142 (2014) p. 312-320.

[43] B.I. Escher, M. Allinson, R. Altenburger, et al., "Benchmarking organic micropollutants in wastewater, recycled water and drinking water with in vitro bioassays" *Environ. Sci. Technol.* 48 (2014) p. 1940-1956.

[44] C. Di Paolo, R. Ottermanns, S. Keiter, et al., "Bioassay battery interlaboratory investigation of emerging contaminants in spiked water extracts – Towards the implementation of bioanalytical monitoring tools in water quality assessment and monitoring" *Water Res.* 104 (2016) p. 473-484.

[45] T. Grummt, J. Kuckelkorn, A. Bahlmann, et al., "Tox-Box: securing drops of life - an enhanced health-related approach for risk assessment of drinking water in Germany" *Environ. Sci. Eur.* 25 (2013) p. 27.

[46] C. Kienle, R. Gauch, E. Vermeirssen, et al., "Methoden zur Beurteilung der Wasserqualität anhand von ökotoxikologischen Biotests - Ergebnisse einer Literaturrecherche und einer Expertenbefragung", Schweizerisches Zentrum für angewandte Ökotoxikologie Eawag-EPEL (Ökotoxzentrum) (2015).

[47] T. Grummt, P. Rettberg, P. Waldmann, et al., in: Biosensors for environmental monitoring of aquatic systems, "Adverse effects in aquatic ecosystems: Genotoxicity as a priority measurement" Springer Berlin Heidelberg (2009) p. 187-201.

[48] T. Grummt, T.-B. Seiler, T. Braunbeck, et al., "Editorial: Special Issue “Effect-related evaluation of anthropogenic trace substances - concepts for genotoxicity, neurotoxicity and endocrine effects”" *Environ. Sci. Pollut. Res.* 25 (2018) p. 3945-3950.

[49] G.D.A. Umbuzeiro, M. Heringa, E. Zeiger, in: In vitro environmental toxicology - concepts, application and assessment, "In vitro genotoxicity testing: Significance and use in environmental monitoring" Springer International Publishing, Cham (2017) p. 59-80.

[50] "Water quality - Evaluation of genotoxicity by measurement of the induction of micronuclei - Part 2: Mixed population method using the cell line V79, German version (DIN EN ISO 21427-2:2009-08)", Beuth Verlag (2009).

[51] "German standard methods for the examination of water, waste water and sludge - Sub-animal testing (group T) - Part 3: Determination of the genotype potential of water and waste water components with the umu-test (T 3) (DIN 38415-3:1996-12)", Beuth Verlag (1996).

[52] D. Shakibai, C. Riegraf, L. Moscovici, et al., "Coupling high-performance thin-layer chromatography with bacterial genotoxicity bioreporters" *Environ. Sci. Technol.* 53 (2019) p. 6410-6419.

[53] W. Busch, S. Schmidt, R. Kühne, et al., "Micropollutants in European rivers: A mode of action survey to support the development of effect-based tools for water monitoring" *Environ. Toxicol. Chem.* 35 (2016) p. 1887-1899.

[54] J.B. Legradi, C. Di Paolo, M.H.S. Kraak, et al., "An ecotoxicological view on neurotoxicity assessment" *Environ. Sci. Eur.* 30 (2018) p. 46.

[55] G. Qu, J. Shi, T. Wang, et al., "Identification of Tetrabromobisphenol A diallyl ether as an emerging neurotoxicant in environmental samples by bioassay-directed fractionation and HPLC-APCI-MS/MS" *Environ. Sci. Technol.* 45 (2011) p. 5009-5016.

[56] G.L. Ellman, K.D. Courtney, V. Andres jr, et al., "A new and rapid colorimetric determination of acetylcholinesterase activity" *Biochem. Pharmacol.* 7 (1961) p. 88-95.

[57] J.J. Menn, J.B. McBain, M.J. Dennis, "Detection of naturally occurring cholinesterase inhibitors in several crops by paper chromatography" *Nature* 202 (1964) p. 697-698.

[58] E.J. Okello, R. Leylabi, G.J. McDougall, "Inhibition of acetylcholinesterase by green and white tea and their simulated intestinal metabolites" *Food Funct.* 3 (2012) p. 651-661.

[59] A. Marston, J. Kissling, K. Hostettmann, "A rapid TLC bioautographic method for the detection of acetylcholinesterase and butyrylcholinesterase inhibitors in plants" *Phytochem. Anal.* 13 (2002) p. 51-54.

[60] M.B. Colovic, D.Z. Krstic, T.D. Lazarevic-Pasti, et al., "Acetylcholinesterase inhibitors: Pharmacology and toxicology" *Curr. Neuropharmacol.* 11 (2013) p. 315-335.

[61] L.G. Costa, G. Giordano, M. Guizzetti, et al., "Neurotoxicity of pesticides: a brief review" *Front Biosci* 13 (2008) p. 1240-1249.

[62] B.R. Pinho, F. Ferreres, P. Valentão, et al., "Nature as a source of metabolites with cholinesterase-inhibitory activity: an approach to Alzheimer's disease treatment" *J. Pharm. Pharmacol.* 65 (2013) p. 1681-1700.

[63] A.P. Murray, M.B. Faraoni, M.J. Castro, et al., "Natural AChE inhibitors from plants and their contribution to Alzheimer's disease therapy" *Curr. Neuropharmacol.* 11 (2013) p. 388-413.

[64] M. Pohanka, "Cholinesterases, a target of pharmacology and toxicology" *Biomed. Pap.* 155 (2011) p. 219-223.

[65] S. Könemann, R. Kase, E. Simon, et al., "Effect-based and chemical analytical methods to monitor estrogens under the European Water Framework Directive" *TrAC, Trends Anal. Chem.* (2018) p. 225-235.

[66] J. Legler, C.v.d. Brink, A. Brouwer, et al., "Assessment of (anti)oestrogenic compounds using a stably transfected luciferase reporter gene assay in the human T47-D breast cancer cell line" *Organohalogen Compd.* 37 (1998) p. 265-268.

[67] E.J. Routledge, J.P. Sumpter, "Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen" *Environ. Toxicol. Chem.* 15 (1996) p. 241-248.

[68] M. Hecker, H. Hollert, R. Cooper, et al., "The OECD validation program of the H295R steroidogenesis assay: Phase 3. Final inter-laboratory validation study" *Environ. Sci. Pollut. Res.* 18 (2011) p. 503-515.

[69] C. Schmitt, G. Streck, M. Lamoree, et al., "Effect directed analysis of riverine sediments - The usefulness of Potamopyrgus antipodarum for in vivo effect confirmation of endocrine disruption" *Aquat. Toxicology* 101 (2011) p. 237-243.

[70] M.B. Müller, C. Dausend, C. Weins, et al., "A new bioautographic screening method for the detection of estrogenic compounds" *Chromatographia* 60 (2004) p. 207-211.

[71] D. Spira, G. Reifferscheid, S. Buchinger, "Combination of high-performance thin-layer chromatography with a specific bioassay - A tool for effect-directed analysis" *J. Planar Chromatogr.--Mod. TLC* 26 (2013) p. 395-401.

[72] D. Schick, W. Schwack, "Planar yeast estrogen screen with resorufin-β-d-galactopyranoside as substrate" *J. Chromatogr. A* 1497 (2017) p. 155-163.

[73] I. Klingelhöfer, G.E. Morlock, "Sharp-bounded zones link to the effect in planar chromatography-bioassay-mass spectrometry" *J. Chromatogr. A* 1360 (2014) p. 288-295.

[74] A. Schoenborn, P. Schmid, S. Bräm, et al., "Unprecedented sensitivity of the planar yeast estrogen screen by using a spray-on technology" *J. Chromatogr. A* 1530 (2017) p. 185-191.

[75] OECD, "Test No. 432: In Vitro 3T3 NRU Phototoxicity Test", OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris (2019).

[76] J. van Meerloo, G.J.L. Kaspers, J. Cloos, in: Cancer cell culture: Methods and protocols, "Cell sensitivity assays: The MTT assay" Humana Press, Totowa, NJ (2011) p. 237-245.

[77] T. Mosmann, "Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays" *J. Immunol. Methods* 65 (1983) p. 55-63.

[78] G.P. Dimenna, B.E. Walker, L.B. Turnbull, et al., "Thin-layer bioautographic assay for Salinomycin in chicken liver" *J. Agric. Food Chem.* 34 (1986) p. 472 - 474.

[79] E.M. Grzelak, B. Majer-Dziedzic, I.M. Choma, et al., "Development of a novel direct bioautography-thin-layer chromatography test: Optimization of growth conditions for gram-positive bacteria, Bacillus subtilis" *J. AOAC Int.* 96 (2013) p. 386-391.

[80] M. Jamshidi-Aidji, G.E. Morlock, "Bioprofiling of unknown antibiotics in herbal extracts: Development of a streamlined direct bioautography using Bacillus subtilis linked to mass spectrometry" *J. Chromatogr. A* 1420 (2015) p. 110-118.

[81] W. Jesionek, E. Grzelak, B. Majer-Dziedzic, et al., "Thin-layer chromatography - Direct bioautography for the screening of antimicrobial properties of plant extracts" *J. Planar Chromatogr.--Mod. TLC* 26 (2013) p. 109-113.

[82] I. Choma, W. Bak, E. Grzelak, et al., "Determination of enrofloxacin and ciprofloxacin in milk by direct bioautography detection" *CBS* 106 (2011) p. 2-4.

[83] S. Parvez, C. Venkataraman, S. Mukherji, "A review on advantages of implementing luminescence inhibition test (Vibrio fischeri) for acute toxicity prediction of chemicals" *Environ. Int.* 32 (2006) p. 265-268.

[84] M. Abbas, M. Adil, S. Ehtisham-ul-Haque, et al., "Vibrio fischeri bioluminescence inhibition assay for ecotoxicity assessment: A review" *Sci. Total Environ.* 626 (2018) p. 1295-1309.

[85] "Water quality - Determination of the inhibitory effect of water samples on the light emission of Vibrio fischeri (Luminescent bacteria test) - Part 3: Method using freeze-dried bacteria (German version of ISO 11348-3:2007)", Beuth Verlag (2009).

[86] G. Eberz, H.-G. Rast, K. Burger, et al., "Bioactivity screening by chromatography-bioluminescence coupling" *Chromatographia* 43 (1996) p. 5-9.

[87] Á.M. Móricz, T.T. Häbe, A. Böszörményi, et al., "Tracking and identification of antibacterial components in the essential oil of Tanacetum vulgare L. by the combination of high-performance thin-layer chromatography with direct bioautography and mass spectrometry" *J. Chromatogr. A* 1422 (2015) p. 310-317.

[88] V. Baumgartner, C. Hohl, U. Hauri, "Bioactivity-based analysis of sunscreens using the luminescent bacteria Vibrio fischeri" *J. Planar Chromatogr.--Mod. TLC* 22 (2009) p. 19-23.

[89] A. Klöppel, W. Grasse, F. Brümmer, et al., "HPTLC coupled with bioluminescence and mass spectrometry for bioactivity-based analysis of secondary metabolites in marine sponges" *J. Planar Chromatogr.--Mod. TLC* 21 (2008) p. 431-436.

[90] W. Schulz, W. Seitz, S.C. Weiss, et al., "Use of Vibrio fischeri for screening for bioactivity in water analysis" *J. Planar Chromatogr.--Mod. TLC* 21 (2008) p. 427-430.

[91] A. Logemann, M. Schafberg, B. Brockmeyer, "Using the HPTLC-bioluminescence bacteria assay for the determination of acute toxicities in marine sediments and its eligibility as a monitoring assessment tool" *Chemosphere* 233 (2019) p. 936-945.

[92] I. Eide, G. Neverdal, B. Thorvaldsen, et al., "Toxicological evaluation of complex mixtures by pattern recognition: correlating chemical fingerprints to mutagenicity" *Environ. Health Perspect.* 110 (2002) p. 985-988.

[93] I. Eide, G. Neverdal, B. Thorvaldsen, et al., "Toxicological evaluation of complex mixtures: fingerprinting and multivariate analysis" *Environ. Toxicol. Pharmacol.* 18 (2004) p. 127-133.

[94] E.L. Schymanski, M. Bataineh, K.U. Goss, et al., "Integrated analytical and computer tools for structure elucidation in effect-directed analysis" *TrAC, Trends Anal. Chem.* 28 (2009) p. 550-561.

[95] J. Hollender, E.L. Schymanski, H.P. Singer, et al., "Nontarget screening with high resolution mass spectrometry in the environment: Ready to go?" *Environ. Sci. Technol.* 51 (2017) p. 11505-11512.

[96] A. Kruve, "Semi-quantitative non-target analysis of water with liquid chromatography/high-resolution mass spectrometry: How far are we?" *Rapid Commun. Mass Spectrom.* 33 (2019) p. 54-63.

[97] J. Guo, W. Shi, Q. Chen, et al., "Extended virtual screening strategies to link antiandrogenic activities and detected organic contaminants in soils" *Environ. Sci. Technol.* 51 (2017) p. 12528-12536.

[98] T. Bader, W. Schulz, K. Kümmerer, et al., "LC-HRMS data processing strategy for reliable sample comparison exemplified by the assessment of water treatment processes" *Anal. Chem.* 89 (2017) p. 13219-13226.

[99] S.C. van der Linden, A.R.M. von Bergh, B.M.A. van Vught-Lussenburg, et al., "Development of a panel of high-throughput reporter-gene assays to detect genotoxicity and oxidative stress" *Mutat. Res., Genet. Toxicol. Environ. Mutagen.* 760 (2014) p. 23-32.

[100] D.R. Stoll, X. Li, X. Wang, et al., "Fast, comprehensive two-dimensional liquid chromatography" *J. Chromatogr. A* 1168 (2007) p. 3-43.

[101] M. Gilar, A.E. Daly, M. Kele, et al., "Implications of column peak capacity on the separation of complex peptide mixtures in single- and two-dimensional high-performance liquid chromatography" *J. Chromatogr. A* 1061 (2004) p. 183-192.

[102] N. Egetenmeyer, S.C. Weiss, "Investigations for the detection of genotoxic substances on TLC plates" *J. Liq. Chromatogr. Relat. Technol.* 40 (2017) p. 69-74.

[103] C. Riegraf, G. Reifferscheid, S. Belkin, et al., "Combination of yeast-based in vitro screens with high-performance thin-layer chromatography as a novel tool for the detection of hormonal and dioxin-like compounds" *Anal. Chim. Acta* 1081 (2019) p. 218-230.

Publications

Publication 1: L. Stütz, S.C. Weiss, W. Schulz, W. Schwack, R. Winzenbacher, Selective two-dimensional effect-directed analysis with TLC, *Journal of Chromatography A* 1524 (2017) 273-282, https://doi.org/10.1016/j.chroma.2017.10.009.

Publication 2: L. Stütz, P. Leitner, W. Schulz, R. Winzenbacher, Identification of genotoxic transformation products by effect-directed analysis with high-performance thin-layer chromatography and non-target screening, *Journal of Planar Chromatography - Modern TLC* 32 (2019) 3, 173-182, https://doi.org/10.1556/1006.2019.32.3.1.

Publication 3: L. Stütz, W. Schulz, R. Winzenbacher, Identification of acetylcholinesterase inhibitors in water by combining two-dimensional HPTLC and high-resolution mass spectrometry, *Journal of Chromatography A*, 1624 (2020) 461239, https://doi.org/10.1016/j.chroma.2020.461239.

Publication 1

L. Stütz, S.C. Weiss, W. Schulz, W. Schwack, R. Winzenbacher, Selective two-dimensional effect-directed analysis with TLC, *Journal of Chromatography A* 1524 (2017) 273-282, https://doi.org/10.1016/j.chroma.2017.10.009.

Journal of Chromatography A, 1524 (2017) 273–282

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Full length article

Selective two-dimensional effect-directed analysis with thin-layer chromatography

Lena Stütz[a,b,*], Stefan C. Weiss[a], Wolfgang Schulz[a], Wolfgang Schwack[b], Rudi Winzenbacher[a]

[a] Laboratory for Operation Control and Research, Zweckverband Landeswasserversorgung, Am spitzigen Berg 1, 89129 Langenau, Germany
[b] Institute of Food Chemistry, University of Hohenheim, Garbenstraße 28, 70599 Stuttgart, Germany

ARTICLE INFO

Article history:
Received 11 May 2017
Received in revised form 17 August 2017
Accepted 4 October 2017
Available online 6 October 2017

Keywords:
Two-dimensional separation
Acetylcholinesterase inhibition assay
Elution head-based extraction
Peak capacity
Orthogonality

ABSTRACT

There are thousands of organic trace substances in the environment that are not fully characterized, and evaluation of their relevance to the ecosystem is difficult. Effect-directed analysis (EDA) is a suitable tool to assess the effects of a substance via in-vitro bioassays, which can provide information about the relevance of the substance. High-performance thin-layer chromatography (HPTLC) has been shown to be a good method for fractionation. Environmental samples, however, often have high complexity, which is why the peak capacity of HPTLC is not sufficient. Therefore, this study focused on the development of selective two-dimensional (2D) HPTLC-EDA to increase the peak capacity and facilitate the identification of effective compounds. Thus, only effective zones were selected in the first dimension in terms of heart-cutting and were transferred to the second dimension through elution head-based extraction. Three 2D approaches were developed and validated. The best results in terms of peak capacity and orthogonality were achieved when the retardation factors of the first dimension were used to adjust the mobile phase (MP) for the second dimension. Applying the acetylcholinesterase (AChE) inhibition assay as an example EDA, analysis of spiked surface water by 2D HPTLC-EDA allowed zones with neurotoxic effects to responsible substances to be assigned. The 2D separation reduced the complexity of effective zones and thus facilitated the subsequent identification of effective compounds. Knowledge about a substanceís effects enabled assessment of its relevance to the environment.

1. Introduction

Because of human influences, the environment gets increasingly polluted with anthropogenic trace substances in addition to naturally occurring ones [1]. Many of these substances have not been characterized, and their impact on humans and the ecosystem has not been sufficiently studied [2]. The challenge is to evaluate the relevance of these various contaminants and to select the important ones. Therefore, effect-directed analysis (EDA) – a combination of physicochemical fractionation, bio-testing and chemical analysis – is a suitable technique [3]. Through fractionation of pollutants in complex samples it is possible to assign bioactive substances to the detected effects in a certain end point [4].

For volatile compounds, gas chromatography coupled with mass spectrometry (GC–MS) is used for EDA because of the good peak capacity and the wide availability of mass spectral databases [5]. However, the limitation of GC with thermo-labile and non-volatile compounds and the challenge to trap the compounds from gaseous phase after separation enables the use of high-performance liquid chromatography (HPLC), which gathers broader substance diversity. Today, HPLC is a frequently used separation technique for EDA [6]. High-performance thin-layer chromatography (HPTLC) has proven to be a particularly suitable separation technique for EDA [7,8]. In contrast to column chromatography, HPTLC is an open separation system, and since the layer is solvent-free after separation, an in-vitro bioassay can be applied directly [9]. It is also possible to vary the applied sample volume over a wide range to increase the sensitivity of the method. Identification of effective compounds is realizable through coupling to a mass spectrometer (MS) or a nuclear magnetic resonance spectrometer (NMR) [10,11].

* Corresponding author at: Laboratory for Operation Control and Research, Zweckverband Landeswasserversorgung, Am spitzigen Berg 1, 89129 Langenau, Germany.
E-mail addresses: Stuetz.L@lw-online.de (L. Stütz), Weiss.S@lw-online.de (S.C. Weiss), Schulz.W@lw-online.de (W. Schulz), Wolfgang.Schwack@uni-hohenheim.de (W. Schwack), Winzenbacher.R@lw-online.de (R. Winzenbacher).

https://doi.org/10.1016/j.chroma.2017.10.009
0021-9673/

However, despite all these advantages, the main disadvantage of HPTLC is the lower peak capacity compared to HPLC [6].

For improvement of HPTLC peak capacity, one-dimensional (1D) gradient development was already shown to be a good strategy [12,13]. In that method, the plate is developed in a stepwise manner with increasing migration distances and different solvent composition within each step. For environmental samples, the peak capacity of such gradient development is often insufficient, which is why a two-dimensional (2D) separation strategy was developed in this study. Many studies have described comprehensive 2D HPTLC where the complete sample was separated on the same plate but in two directions [14–16], but a selective 2D HPTLC-EDA is presented here for the first time. Also a study about 2D HPLC-AChE was identified, where a 2D on-line HPLC separation was applied to sewage water. But the main difference of the study to our one is, that the bioassay was not applied in two dimensions but only after the second separation [17]. We used the elution head-based extraction technique to transfer effective zones from the first to the second dimension [18]. Thereby the elution head is pressed to the layer to seal the zone which should be extracted. The elution solvent flows over the sealed zone and washes out the compounds, where a frit in the elution head restrains solid components from the layer and matrix [19]. After the separation in each dimension, the acetylcholinesterase (AChE) inhibition assay, an enzyme test for the detection of neurotoxic effects, was used [20]. The coupling of HPTLC and AChE assay is applied frequently for investigation of acetylcholinesterase inhibiting compounds in plant extracts [21]. But the assay procedure is quite different depending on the user. A significant variation is the application of AChE onto the HPTLC plate. This is done either by spraying [22,23] or by immersing the plate in the enzyme solution [24]. Various substrates are also used, e.g. 1-naphtyl acetate or 5,5′-dithiobis-(2-nitrobenzoic acid) [21]. For the present study, the plate was immersed in the AChE solution and 3-indoxyl-3-acetate was used as substrate. With our developed workflow, only effective zones were transferred to the second dimension so that substances without any effect were already excluded after the first dimension. Through the second separation, a further fragmentation of the effective zones could be achieved with the aim of prioritizing effective compounds of interest. The next important step was the coupling of this method to further analytical techniques such as MS or NMR with the aim of identifying the prioritized effective compounds.

2. Materials and methods

2.1. Chemicals and reagents

Ultrapure water was received with an ultrapure apparatus (Purelab Ultra, Elga LabWater, Lane End, UK). Acetonitrile (Rotisolv ≥ 99.95 %, LC–MS-Grade), 2-propanol (Rotisolv ≥ 99.95 %, LC–MS-Grade) and acetone (Rotisolv ≥ 99.9 %, HPLC) were purchased from Carl Roth (Karlsruhe, Germany). Dichloromethane and *n*-hexane were purchased from J.T. Baker (Center Valley, USA). Methanol (AMD Chromasolv ≥ 99.9%) was supplied by Sigma-Aldrich (Steinheim am Albuch, Germany). Chloroform (HiPerSolv Chromanorm) and ammonia (25%, AnalaR Normapur) were purchased from VWR International (Bruchsal, Germany), and formic acid (98–100%), ethyl acetate (Uvasol) and toluene (Uvasol) were supplied by Merck (Darmstadt, Germany).

For the AChE inhibition assay, acetylcholinesterase Type VI-S (2000 U/vial) from electric eel and ascorbic acid and 3-indoxyl-3-acetate from Sigma-Aldrich were used. Dimethyl sulfoxide, bovine serum albumin, hydrochloric acid (32%) and 2-amino-2-(hydroxymethyl)-1,3-propanediol were supplied by Merck (Darmstadt, Germany).

2.2. Standards

Pure standard substances were acquired from various suppliers (Carl Roth, Karlsruhe, Germany; LGC Standards, Teddington, UK; Merck, Darmstadt, Germany; Sigma-Aldrich, Steinheim am Albuch, Germany; Thermo Fischer, Karlsruhe, Germany; VWR International, Bruchsal, Germany). Standard stock solutions were prepared by dissolving 10 mg of each pure substance in 100 mL methanol and storing at −18 °C. To prepare respective standard solutions, the stock standards were diluted in methanol to the required concentration. Standard solutions were used for four weeks.

For mobile phase (MP) development, five standard solutions (A–E) with 5–8 compounds (10 ng/μL) were used. Their exact composition is given in Fig. 2. To gather a possibly broad retention area the substances were chosen according to their polarity. The $\log K_{ow}$ values were determined using the KOWWIN software (v1.68) from US EPA. For the 35 model compounds the middle value of $\log K_{ow}$ was 3.0 with a range from 0.1 (cefixime) up to 6.1 (bromophos-ethyl). The stability of chromatography was examined with a mix consisting of nine substances (1,3,6-naphthalenesulfonic acid, 1,5-naphthalenesulfonic acid, 1-naphthalenesulfonic acid, theobromine, caffeine, thiourea, *N*-phenylacetamide, benzamide, and *N*,N-dimethyl-4-[(*E*)-phenyldiazenyl]aniline) with 10 ng/μL [8]. A multi-component standard (83 μg/L) with 448 environmentally relevant anthropogenic trace substances, such as pharmaceuticals, herbicides, X-ray contrast agents, and others, was used for the determination of a frequency distribution across the retardation factors (hR_F). The spiking of surface water was performed with a mix consisting of 50 neurotoxic substances (Table S1).

2.3. Sampling and sample preparation

A grab sample of surface water was taken from the river Danube (Leipheim, Germany). The sample (1 L) was spiked with the neurotoxic mix to a final concentration of 4 μg/L. Enrichment was performed by solid phase extraction (SPE) with an enrichment factor of 1000.

Enrichment was performed by solid phase extraction (SPE) with Bond Elut Plexa cartridges (Agilent Technologies, Santa Clara, USA) containing a non-polar divinylbenzene-based neutral polymeric sorbent. The cartridges were conditioned with each 5 mL *n*-hexane, dichloromethane, acetone, methanol, and ultrapure water. The sample was filled in a storage vessel and ran over the cartridge, powered by a peristaltic pump with a flow of 2.5 mL/min. After loading, the cartridges were dried for 45 min in an airstream. Three solvents were used for elution. At first, 3 mL methanol (0.4% v/v ammonia) was used. The second eluent consisted of a 50:50 v/v mixture of methanol (0.4% v/v ammonia)-ethyl acetate (4 mL), and the third eluent was dichloromethane (3 mL). Evaporation was performed in a gentle stream of nitrogen, and 50 μL of ultrapure water was added as keeper before evaporation of dichloromethane. The residue was diluted with methanol to a volume of 1 mL s conditioned and eluted the same way the sample cartridge was treated but without any contact with a sample.

Recovery rates of SPE were determined for the 35 model compounds used for method development. The substances were spiked to deionized water before and after solid phase extraction to get the same matrix in both samples. Concentrations were chosen that 100% recovery would lead to 10 μg/L after the enrichment. Measurement was done via HPLC-HRMS (see Chapter 2.4). The median of recovery achieved 87.5% with a 10% quantile of 46.1%. One substance could not be detected in the positive ESI mode, why it was not considered (cefixime). With that a medium enrichment factor of 875 could be calculated.

2.4. Apparatus and software

For application, separation and detection, the Automatic TLC Sampler 4 (ATS 4), Automatic Developing Chamber 2 (ADC 2), Automated Multiple Development 2 (AMD 2), TLC Scanner 3 and TLC Visualizer were used. The associated software was winCATS (1.4.9). Elution of zones from the plate was performed with the TLC–MS Interface 2 with an oval elution head (4 × 2 mm) (all from CAMAG, Muttenz, Switzerland).

For application of the AChE inhibition test, the Chromatogramm Immersion Device 3 (CAMAG), the spraying apparatus, ChromaJet DS 20 (Desaga, Wiesloch, Germany), and an incubator at 37 °C (VWR International) were required.

The determination of the frequency distribution of the multi-component standard across the hR_F values and also the determination of SPE and extraction recoveries were performed via HPLC-HRMS analysis with the LC20 series (Shimadzu, Kyoto, Japan) coupled to the Quadrupol-Time-of-flight/mass spectrometer (Q-TOF/MS), TripleTOF 5600, from Sciex (Framingham, USA). For ionization, electrospray ionization (DuoSpray) in the positive mode was used. Gradients and parameters were adjusted according to the study of Bader et al. [25]. Data acquisition was performed with Analyst TF software (1.7). For peak integration, the MultiQuant software package (3.0.2) was used. For each of the 448 substances (also includes the 35 model compounds used for method development) a MS/MS spectrum was recorded and retention time was determined. So the substance identification (min. 2 fragments) could be achieved through a library search via the software tool LibraryView (Sciex, Framingham, USA).

2.5. Selective 2D HPTLC-EDA

HPTLC LiChrospher silica gel 60 F_{254S} plates (Merck) were immersed twice in 2-propanol for 20 min. After drying at 120 °C on the plate heater, the plates were predeveloped to the top edge with acetonitrile. Finally, the plates were heated again to 120 °C for 20 min. Cleaned plates were stored in a desiccator at room temperature. This procedure was necessary because otherwise the dirt of the plates gathers in streaks over the whole plate after separation and disturbs the bioassay detection.

Application of standards and samples was performed with the ATS 4 as 6 mm bands 10 mm from the lower edge. For development, two apparatuses were used. The ADC 2 was used for isocratic, one step separations, and the AMD 2 was used for separations with more than one development step. In the ADC 2, 1 min was chosen as the duration for the humidity setting. The chamber saturation amounted to 15 min, preconditioning time was set to 30 s, the migration distance came to 70 mm, and the drying time amounted to 20 min. The migration distance of the gradient development with 16 steps was 80 mm. The AMD 2 was rinsed once before use. The minimum end pressure was set to 10 mbar, and the maximum pressure increase allowed was 10 mbar/min.

The selective extraction was performed by choosing only effective zones of the first dimension to be eluted with the TLC–MS interface. Therefore, an additional separation of the sample on a new plate was necessary, and the target zones were located by the coordinates after the first in-vitro assay. With methanol as the extraction eluent, the substances in the target zone were eluted for 1 min with a flow of 200 μL/min [26]. To avoid a carryover between extractions, the elution head was purged after each extraction with methanol for at least three minutes. To obtain enough extract for separation in the second dimension, the sample was applied three times, and the eluates of the effective zones were collected in a vial and again applied to a plate with the same stationary phase at a volume of 200 μL. After separation in the second dimension with another solvent, the effects were detected by the in-vitro bioassay again (Fig. 1).

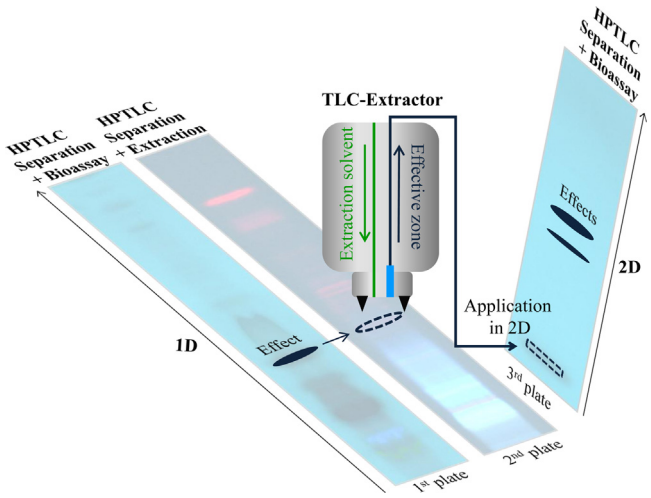


Fig. 1. General workflow of 2D HPTLC-EDA.

Recovery rates of elution head-based extraction were determined for the 35 model compounds used for method development. A mix of these substances (83.3 μg/L) was applied to the plate with 6 × 4 mm and extraction was done without separation in the middle of this area. This procedure was repeated three times. The extracted zones were measured via HPLC-Q-TOF/MS with respectively three injections (10 μL) and by consideration of the elution headís area (7.142 mm^2) substance concentrations could be calculated. The extraction recovery was determined through comparison of signals with an external standard-mix, so matrix effects cannot be excluded.

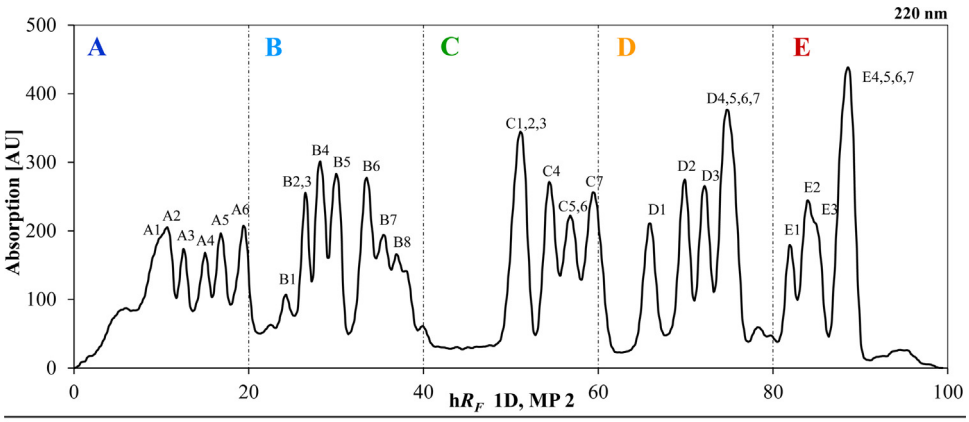
Three different approaches were developed for the 2D chromatography. The composition of all developed MPs can be found in Table S2. Due to the long development times of the gradients, only isocratic separations were considered for the second dimension:

- Isocratic approach: Both dimensions were developed with isocratic MPs.
- Gradient approach: A gradient development was used in the first dimension, whereas the same isocratic MP as the isocratic approach was used for separation in the second dimension.
- Dynamic approach: The gradient development of the gradient approach was used for the first dimension, and several MPs for separation in the second dimension were developed. These MPs were adjusted on the hR_F in the first dimension. Five arbitrary retardation areas were appointed in equal intervals: A: [0–20], B:]20–40], C:]40–60], D:]60–80] and E:]80–100], and for each of these areas a different MP was developed. Therefore, the elution strength could be exactly adjusted to the substances that were investigated.

The results were documented with the TLC-Visualizer (254 nm, 366 nm and white light) and with the TLC-Scanner as a multiple wavelength scan (7 wavelengths from 190–300 nm).

2.6. Acetylcholinesterase inhibition assay with HPTLC

Before application of the biological assay the plate has to be adjusted to a neutral pH. Therefore the plates, which were developed with acidic mobile phases (MP 1, MP 2 and MP A) were placed for 10 s in NH_3 vapor. Afterwards the plates were put under vacuum (10 min) to remove the remaining NH_3 (pH after neutralization approx. 7.5).



No.	A	B	C	D	E
1	Amisulpride N-oxide	Benazolin	Dimefuron	Estrone	Phosalone
2	Iodipamide	Fenoxaprop	Myclobutanil	Boscalid	Aclonifen
3	Cefixime	Ketoprofen	Phenacetin	1-(2-Chloro-phenyl)-3-phenylurea	Fenthion
4	Iotalamic acid	Triclopyr	Fenarimol	Diflubenzuron	Dichlofenthion
5	Picloram	4´Hydroxy-diclofenac	2,6-Dichloro-benzamide	Methiocarb	Iodofenphos
6	Clopyralid	Acetylsulfamethoxazol	Chlorotoluron	Alachlor	Bromophos-ethyl
7		Chloramphenicol	Norflurazon	Profenophos	Profluralin
8		Pentoxifylline			

Fig. 2. Separation of 35 compounds with MP 2 in the first dimension of the dynamic approach.

The AChE inhibition assay described in Weins and Jork was used [27] with several modifications. AChE was dissolved in 0.05 M Tris-HCl buffer (pH 7.8). For stabilization of the enzyme on the Plate 0.1% v/v bovine serum albumin was added. In contrast to Weins and Jork, we used 3-idoxyl-3-acetate as a substrate to evaluate the activity of the enzyme. Therefore, 0.5 g/L 3-indoxyl-3-acetate was dissolved in an aqueous solution of 4% v/v DMSO. If the enzyme was active, the substrate was cleaved to indoxyl, which reacted with oxygen to indigo white. Both products could be measured because of their blue fluorescence at 366 nm.

The AChE inhibition test began with the immersion of the plate in the enzyme solution (Chromatogramm Immersion Device 3, dipping time 2 s, dipping speed 2 cm/s). The supernatant enzyme solution was carefully pulled off with a squeegee. The plate was incubated for 5 min in an incubator at 37 °C with humidity higher than 90 %. After 6.5 min, the substrate solution was sprayed to the plate with the spraying apparatus from Desaga (amount of reagent: 580 µL, distribution: 0.03 µL/mm^2). The plate was placed in the TLC Visualizer, and 9 min after the immersion, the detection of enzyme inhibition started. A wavelength of 366 nm was chosen, and a sequence capturing 10 pictures in 10 min was started. The exposure time was set to 800 ms. For the evaluation, the second picture was used. Paraoxon-ethyl and methiocarb-sulfoxide were used as positive controls and therefore applied to each plate in a total amount of 20 ng/band and 10 ng/band. After the bioassay the effects of the substances were evaluated [28] and used as quality control. Paraoxon-ethyl achieved an effect of 47% (SD = 3.9%, N = 40), where methiocarb-sulfoxide inhibited AChE to 40% (SD = 2.1%, N = 40).

2.7. Calculation of analytical parameters

2.7.1. Orthogonality $O_\perp$

To evaluate the effectiveness of the 2D HPTLC separations, different analytical parameters were calculated, and the first one was the orthogonality. A 2D separation was considered orthogonal if the current hR_F of the compounds were completely independent. For calculation of orthogonality, a procedure called bin counting published by Gilar et al. [29] was used. The following formula to determine the orthogonality ($O_\perp$) of LC x LC separations was derived:

$$O_\perp = \left(\sum bins - \sqrt{P_{max}}\right) / 0.63 \cdot P_{max} \quad (1)$$

Briefly, P_{max} gives the number of all bins that were equal to the number of measured compounds. The sum of the bins describes the total number of occupied bins after 2D development. Here, the area between hR_F = 0–100 was divided into bins in both directions of the development according to the number of substances that were separated.

2.7.2. Practical peak capacity N_p

The second analytical parameter investigated was the practical peak capacity (N_p). The peak capacity (P) represents the maximum number of compounds that could theoretically be separated within a specific migration range. The peak capacity of a 1D separation was calculated as the quotient of the effective analytical distance (distance between the first and the last peak) and the peak base width (4σ). If the separation methods of a 2D approach were completely orthogonal, the peak capacities could be multiplied. In reality, this case does not occur, and the area of coverage has to be considered. The practical peak capacity (N_p) could be calculated as [29]:

$$N_p = P_{1D} \cdot P_{2D} \cdot (0.63 \cdot O_\perp + 1/\sqrt{P_{max}}) \quad (2)$$

P_{1D} and P_{2D} are the peak capacities of the first and the second dimension whereas the area coverage is obtained from the quotient of the summed bins and P_{max}.

2.7.3. Eluent strength ε_{AB}

The procedure for estimation of eluent strength (ε_{AB}) was previously described [30] and used here with a few restrictions. First of all, there was no value for solvent strength (ε_0) for formic acid. For acetic acid, ε_0 was given as »1, whereas the ε_0 of formic acid was estimated to be 1.2. Through this estimation, changes to the true eluent strength of MP A could be calculated. However, formic acid

was only used in low volume concentrations in the MP A, which was why this change was deemed to be negligible. Because of the changing solvent system constitutions after each step, no eluent strength could be calculated for gradient development.

3. Results and discussion

3.1. Development of selective 2D separations with HPTLC

First of all, an isocratic 2D approach with constant composition of the MP was developed. Formic acid-acetonitrile-dichloromethane (1:50:49, v/v/v, MP 1) was used as the solvent for the first dimension, and the second dimension was separated with methanol-chloroform (20:80, v/v, MP B). This MP B was applied because of its medium eluent strength of 0.56 to gather both the polar and the nonpolar compounds in a sample. The total time for the two isocratic separations amounted to 80 min.

For improvement of the peak capacity of the first dimension, gradient development (automated multiple development, AMD) with 16 steps was applied (gradient approach). The plate was developed in a stepwise manner with increasing migration distances. Each step consisted of another solvent composition with decreasing solvent strength (Table S2). The first three separation steps were necessary to focus the substances on the application zone to a thin band. As solvents, methanol (0.05% v/v formic acid), dichloromethane and *n*-hexane were used. For better comparison of the peak capacities of the two approaches, the same solvent as the isocratic approach was used for separation in the second dimension (MP B). This gradient approach required a total time of 215 min.

To further increase the peak capacity, the gradient approach was extended to a dynamic one. Therefore, the retardation area of the first dimension (MP 2, AMD) was divided in five areas (A–E). For each area, another MP was developed (MP A–E) so that the substances were separated in the second dimension in a manner dependent on their hR_F in the first dimension. Thus, after the gradient development in the first dimension, a second gradient development was applied in the second dimension with eluent strengths adjusted to the substanceís hR_F in the first dimension. For the method development of these MPs, 35 substances with known hR_F in the first dimension (MP 2) were used (Fig. 2).

Method development for the dynamic approach was performed on the basis of these 35 compounds with the aim of distributing the selected substances over a preferably broad retardation area. In the end, several substances that had similar hR_F in the first dimension could be separated at baseline after the second dimension (e.g., diflubenzuron D4, methiocarb D5, alachlor D6, profenphos D7). The advantages of the dynamic approach over the gradient approach were shown by comparing the 2D separation of the 35 substances (Fig. 3). Whereas the gradient approach only achieved an improved separation of standards in area B, the dynamic approach led to an obviously broader distribution of the compounds over the whole separation area compared to separation in the first dimension. MP B, which was used as the solvent for the separation in the second dimension, proved to be best qualified for separation of the retardation area from]20–40].

For separation in the second dimension with the dynamic approach, different MPs were developed. The separation with MP C–E showed better results with more than one development step as the whole migration distance of 60 mm was developed. As an example, the first separation step with MP D led to only four fractions distributed over a retardation range of 23. After the third development step, all substances could be almost completely separated over a retardation range of 43.

Different variables influenced the separation of the compounds. One influential variable was the selectivity of the used MP, which became apparent in the alternating order of the compounds after the second separation (e.g., Fig. 3, B, MP C). A further variable was the eluent strength (ε_{AB}), which could be observed by separation with MP E. Though the order of the substances remained the same as during chromatography in the first dimension, they could be separated to a hR_F range from 8 up to 97. The ε_{AB} was calculated for all of the five developed MPs of the dynamic approach (Fig. 3). MP A, developed for retardation area from [0 −20], achieved the highest ε_{AB} (0.65). With increasing hR_F, the ε_{AB} of the developed MPs decreased, which is equivalent to a gradient development in the second dimension. MP B and C achieved ε_{ABs} of 0.56 and 0.53, respectively, and the eluent strengths of MP D and E were 0.40 and 0.23, respectively.

3.2. Investigation of orthogonality and peak capacity

For assessment of the developed 2D separations, the orthogonality was determined using the bin counting method. For each pair of MPs, the hR_F of 25 substances were determined. According to [29], hR_F were normalized with the minimal and maximal retardation factors of each separation to remove void spaces in the 2D separation plot (feature scaling). Then, the number of occupied bins was determined (TablesS3–S5 and Figs S1–S3). Orthogonality of 32% and 25% was reached with the isocratic and the gradient approach, respectively. The something better orthogonality of the isocratic approach could be explained by the fact, that acetonitril (class VI), which is used in the 1D of the isocratic approach, is in another selectivity class [31] than methanol (class II), which is used in the 1D of the gradient approach. The MP B for the second dimension of both approaches consists of methanol-chloroform (20:80, v/v). As was expected, the dynamic approach led to a higher orthogonality compared to the former 2D approaches. For each retardation area, A–E, 25 substances with appropriate retardation factors (1D, MP 2) were selected. Thus, 125 substances in total were measured in the five areas leading to an orthogonality of 65% for the dynamic approach (Table 1).

To compare the effectiveness of the developed approaches, their peak capacities were calculated considering the effective separation distance and the mean peak width (at base) across all 25 substances. First of all, the peak capacities of the two MPs for the separation in the first dimension were considered (MP 1 and 2). The isocratic MP 1 achieved a peak capacity of 19.1, whereas the gradient development reached a peak capacity of 29.9. In comparison, the gradient development lasted four times longer than the isocratic development but resulted in smaller peaks (MP 1: mean peak width 2.9 mm, MP 2: mean peak width 1.6 mm). The width of the elution head was 2 mm, and zones smaller than 2 mm are more suitable for extraction. The width of the elution head limited the maximal achievable peak capacity. A lower peak width than 2 mm did not lead to an improvement in peak capacity because a width of 2 mm was determined through the extraction of zones. This effect only occurred during 2D separation with the gradient development (MP 2) where the zones were smaller than 2 mm. Thus, for 2D separation with the MP 2, a peak capacity of 24.1 (peak width set as 2 mm) was used for the calculations in Table 1. In an ideal orthogonal system, the peak capacities of both dimensions can be multiplied, whereas in reality, the orthogonality of the separation dimensions has to be considered. Because orthogonalities of <100% were always reached, the ideal peak capacities (P) were reduced to practical peak capacities (N_p), which were calculated for each 2D approach (Table 1).

The isocratic and gradient approaches achieved peak capacities of 112.2 and 127.8, respectively. Compared to the dynamic approach with a practical peak capacity of 204.2, they were lower by a factor of 1.6. In summary, the peak capacity could be increased

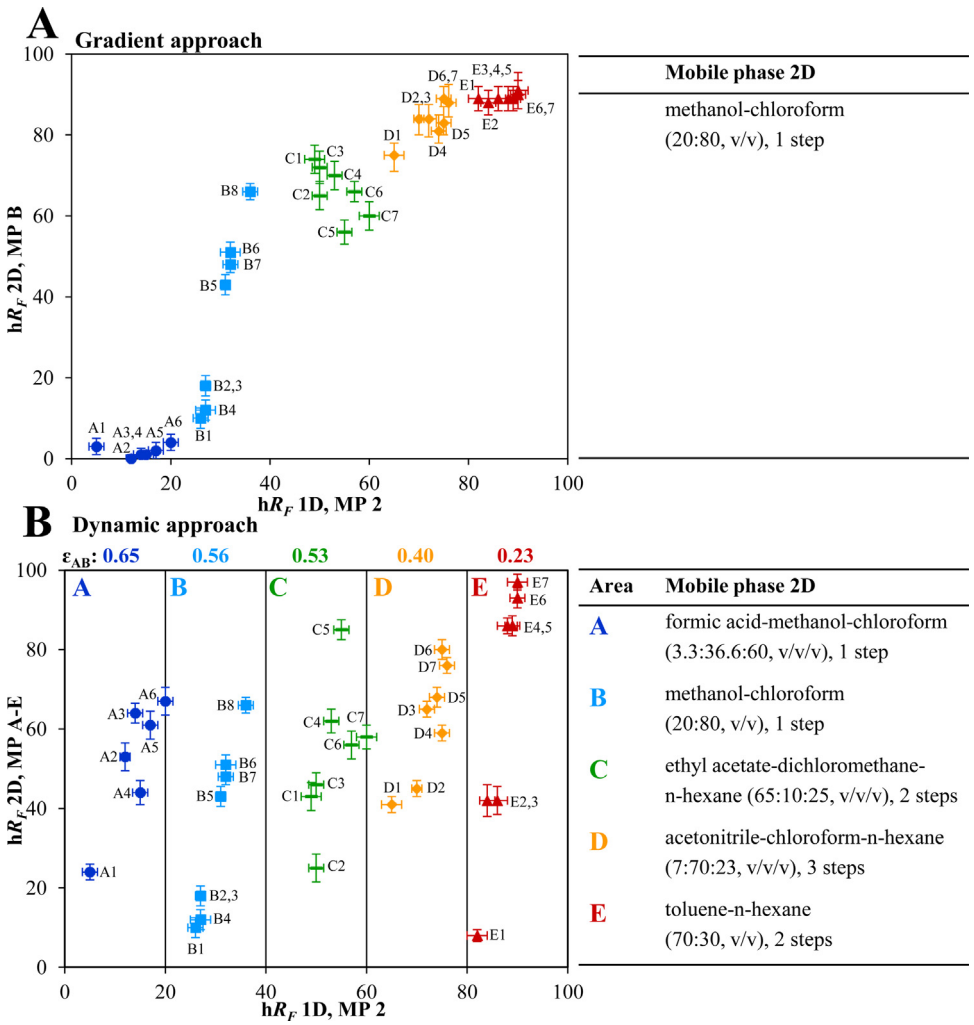


Fig. 3. Comparison of 2D separation of the 35 substances with the gradient approach (A) and the dynamic approach (B). The numerals give the substances in the respective area listed in Fig. 2. The peak width of compounds in both dimensions is given in the error bars. ε_{AB} gives the eluent strength of MP A–E.

Table 1
Peak capacity and orthogonality of developed separation methods.

Separation	Mobile phase (1D - 2D)	Peak capacity (P_{1D} or P_{1D} * P_{2D})	Orthogonality	Practical peak capacity
1D	MP 1	19.1	–	–
	MP 2	29.9	–	–
Isocratic approach	MP 1 - MP B	280.6	0.32	112.2
Gradient approach	MP 2 - MP B	355.0	0.25	127.8
Dynamic approach	MP 2 - MP A - E	411.6	0.65	204.2

by a factor of 6.8 using the dynamic 2D approach compared with the 1D gradient approach (MP 2).

In the literature, peak capacities of 1D HPLC gradient developments are distributed over a wide range from 100 to 1500, whereas with 2D HPLC separations with different stationary phases, peak capacities of several thousand could be achieved [32–35]. When comparing these values with the peak capacities obtained in the present study, 2D HPTLC is still in the lower range of 1D HPLC separation.

These calculated peak capacities for separation can differ slightly from peak capacities by application of the described approaches in EDA. Through application of in-vitro bioassays on the HPTLC plate, diffusion effects may cause zone broadening compared to mere separation. Thus, peak capacity is somewhat decreased depending on the amount of the applied substance and also on the effective power of the substance. The higher the amount per zone and the effective power of the substance, the higher the diffusion effects through detection of the effect. There are substances with only low effects on the applied bioassay. Then, the zone width might be smaller by consideration of the effects compared to the mere separation because only the maximum of the peak exceeded the detection limit and caused an effect in the biological test. To estimate the influence of these effects, the zone widths of five substances were determined before and after the application of the AChE inhibition assay (Table 2). Both the isocratic separation (MP 1) and the gradient development (MP 2) for separation in the first dimension were considered. Three of five substances did not differ in the zone width before and after the AChE inhibition assay. This result can be explained by the fact that the AChE inhibition assay is a very fast assay with a short incuba-

Table 2
Comparison of peak widths before and after the application of the AChE inhibition assay.

Substance (100 ng)	Mobile phase	Peak width before bioassay [mm]	Peak width after bioassay [mm]	Broadening through bioassay [mm]
Aminocarb	1	4.8	4.8	0.0
	2	3.5	3.5	0.0
Azamethiophos	1	5.4	8.4	3.0
	2	2.8	4.9	2.1
Isoprocarb	1	4.2	4.2	0.0
	2	2.8	3.5	0.7
Chlorpyriphos	1	4.2	1.8	−2.4
	2	2.8	1.4	−1.4
Paraoxon	1	4.2	4.2	0.0
	2	2.8	2.8	0.0

tion time (9 min). By application of bioassays with long incubation times of a few hours (e.g., Yeast estrogen screen, 3 h), higher peak broadening is expected.

In this study, a qualitative 2D method for improvement of peak capacity was developed. For review, that the substances generally achieve the second dimension, a recovery test was performed for the 35 model compounds used for method development. This test revealed that the recoveries of substances fluctuate dependent on their solubility in the extraction solvent. For methanol as extraction solvent a medium recovery of 56.0% could be achieved, with a 10% quantile of 39.8%. This determination of recovery was gained without separation of substances. But additionally, the zone width of substances is crucial for recovery because of the given width of the elution head. Peaks wider than 2 mm are only extracted in the middle part, and the peak flank gets lost and recovery decreases.

3.3. Frequency distribution of the dynamic approach

In the dynamic approach, the retardation area of the first dimension was divided into five areas. To confirm the arbitrary division of these areas, a multi-component standard comprising 448 substances was separated, and a qualitative frequency distribution across the five areas was determined. The multi-component standard was separated on the first dimension with MP 2, and then 29 zones from 10 to 80 mm at an interval of 2.5 mm were extracted. The elution head had a width of 2 mm so that 0.5 mm was not extracted, but because of a middle zone width of 2 mm, almost all substances could be gathered. The measurement was performed in triplicate by HPLC-Q-TOF/MS in the positive mode.

Of the 448 substances, 90 compounds were located in the first retardation area A from]0 –20], corresponding to 20 % of substances. One hundred eleven (25%) compounds had hR_F between]20–40], and 21% (94 compounds) of the substances were located in the third retardation area C between]40–60]. In the fourth retardation area D, 26% (118) of all compounds were located. The area that was the least occupied was the fifth area with 33 compounds (7%). Two (iopromide and irgarol-descyclopropyl) of the 448 substances could not be gathered with this investigation.

With these results, it could be shown that the 446 compounds are distributed relatively consistently over the whole retardation area in the first dimension. All areas were occupied by 20–26% of the substances with the exception of the fifth area, E. Through the consistent distribution of compounds in the areas, equal classification was confirmed.

3.4. Investigation of stability of hR_F

Because the extraction of the effective zone was performed on another plate than the bioassay, the stability of hR_F was very important to ensure that the right coordinates were used for the extraction. This issue is also substantial for the comparison of results over a longer period. Therefore, the chromatography control and also the respective mixes A–E were applied to each developed plate. According to the substances that could be separated with the applied MP, 7–9 substances were used as controls, and their hR_F were documented. Then, the standard deviation of each substance for all measurements was determined and averaged for all substances investigated (Table 3). With a mean standard deviation of 1.56, the variation in hR_F between the plates was so small that the coordinates from another development could be used for extraction of effective zones.

Table 3
Stability of retardation factors ($\overline{SD}$ = average standard deviation about all measurements and substances, time period: 6 month).

Dimension	Mobile phase	No. of plates	$\overline{SD}$ of control substances (N = No. of control substances)
1D	1	17	1.28 (N = 7)
	2	41	1.93 (N = 9)
2D	A	12	1.92 (N = 9)
	B	28	1.81 (N = 8)
	C	12	1.24 (N = 7)
	D	8	1.76 (N = 7)
	E	10	1.00 (N = 7)

3.5. Selective 2D HPTLC-AChE inhibition assay with spiked surface water

A sample from the river Danube was investigated with the developed 2D approaches in combination with the AChE inhibition test as the in-vitro bioassay. The sample was spiked with a mix consisting of 50 neurotoxic compounds to show that the developed method was also applicable to other compounds besides the 35 standards used for method development.

Initially, the MPs for the separation in the first dimension (MP 1 and MP 2) were compared (Fig. 4). With the use of gradient development, thinner and sharper zones could be achieved, and the effects in the AChE inhibition assay were separated much better compared to isocratic development with MP 1.

In all approaches, seven effective zones were selected for extraction. Through overlap of zones by separation with MP 1, identification of clearly separated effective zones was difficult. Each zone was extracted three times (200 μL, respectively), and the extract was collected in a vial and mixed. By application of 200 μL to the second dimension, dilution effects could be prevented. For the gradient and dynamic approaches, the same MP was used in the first dimension, for which reason the same zones for extraction were selected.

For the isocratic and gradient approaches, all seven extracted zones were separated in the second dimension with MP B. By application of the dynamic approach, the MPs were chosen dependent on the hR_F of the extracted zones in the first dimension. The hR_F of zone 1 amounted to 24, which is why the development was performed with MP B. MP C was used for separation of zone 2 (hR_F = 54). Zones 3–6 (hR_F = 64, 68, 71 and 78) were separated with MP D, and the seventh zone with an hR_F of 81 was separated with MP E. Finally, the AChE inhibition assay was used (see Fig. 5).

Through comparison of the three approaches, it became obvious that there were very few differences between the isocratic and gradient approaches. The better peak capacity reached by MP 2 compared to MP 1 seems not to be crucial for the final outcome of the 2D HPTLC-EDA in this case. The thinner and sharper zones achieved by use of gradient development were an advantage during extraction of effective zones because they had higher recovery rates. A decisive improvement, however, was achieved

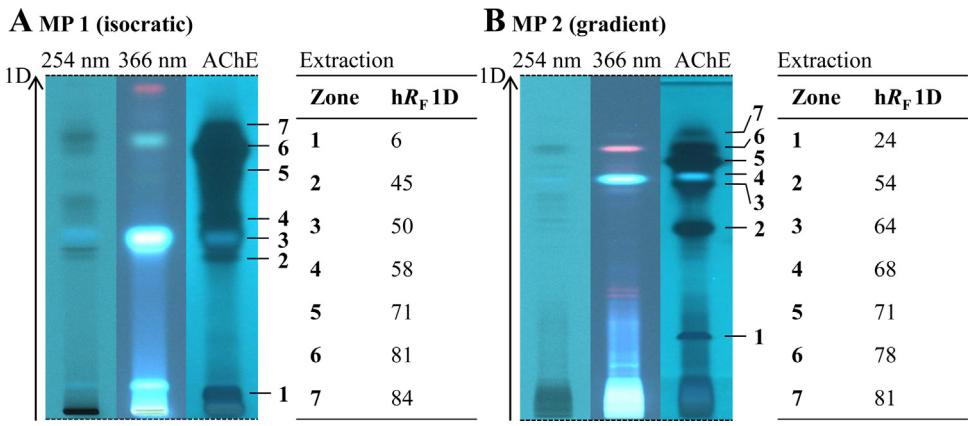


Fig. 4. Investigation of a spiked surface water extract in the first dimension with (A) the isocratic development (MP 1) and (B) the gradient development (MP 2). Neurotoxic effects were detected with the AChE inhibition assay. The hR_F of the extracted zones (1–7) are listed in the tables.

Table 4
Overview of the assignment of effective zones to the spiked substances.

Separation	Effective zones	Zones assigned to one substance	Zones assigned to multiple substances	Zones without assignment
MP 1	7	0	6	1
MP 2	7	1	6	0
Isocratic approach	10	3	3	4
Gradient approach	9	2	6	1
Dynamic approach	17	8	5	4

Table 5
Zones with one matching candidate to hR_F of the first and second dimension.

Approach	Zone	hR_F 1D (±2)	hR_F 2D (±2)	Candidate
isocratic	3.1	50	69	metoxuron
	5.1	71	78	trichlorfon
	5.2	71	85	paraoxon
gradient	1.1	24	54	donepezil
	7.1	81	78	carbosulfan
dynamic	1.1	24	54	donepezil
	2.1	54	75	3-hydroxycarbofuran
	3.2	64	46	methabenzthiazuron
	4.1	68	20	pirimicarb
	4.3	68	47	methabenzthiazuron
	4.4	68	57	acridine
	6.3	78	50	trichlorfon
	6.4	78	74	linuron

with the dynamic approach. With the gradient approach, nine zones could be separated in the second dimension, and with the dynamic approach, 17 zones could be distinguished. Some zones, which could not be separated in the gradient approach, were divided into five zones through the application of the adjusted MPs (Fig. 5, zone 6).

For further description of the separations, the number of spiked substances needs to be reviewed to clearly assign the substances to the detected neurotoxic effects. Therefore, the assignment was exclusively accomplished based on the known hR_F of the 50 spiked substances, which were determined for all MPs (Table S1). Then, for each zone, appropriate substances were searched using the hR_F of the first and second dimension. Thereby, a variation of ±2 was tolerated because this value was determined as the hR_F's average standard deviation during separations (Table 3).

If only the hR_F of the first dimension was considered, just one substance could be unambiguously assigned to an effective zone (donepezil was assigned to the zone 1 by development with MP 2). To all other zones in the isocratic approach with no matching substance, more than one possible candidate could be found. By consideration of the hR_F of both dimensions, 13 candidates could be unambiguously matched to one effective zone. Whereas with the isocratic and the gradient approaches, 3 and 2 candidates were merged to effective zones, respectively, and with the dynamic approach, 8 substances could be clearly assigned to the detected effects (Tables 4 and 5).

In Table 5, all zones with exactly one matching candidate are shown. Additionally, there were some effective zones (N = 10) without any possible assignment of the spiked substances. Either the neurotoxic effects were caused through unknown substances in the surface water itself or the hR_F of the spiked substances varied too much for an assignment. Such false-negative results were confirmed through the allowance of ±4 as tolerable variation of retardation factors, and then only 5 zones could not be matched to a possible candidate. However, the higher tolerance also reduced the clearly assigned substances to 6 so a variation of ±2 was maintained.

For several zones (N = 26), more than one possible candidate could be found. The main reason was co-migrating substances with the same retardation factors in both dimensions. However, the allowed standard deviation of hR_F (±2) could be a cause for false-positive results. This possibility is also reflected through the substance methabenzthiazuron with a hR_F of 66 by use of MP 2. By allowing ±2 variation, the substance fit to zone 3.2 and also to zone 4.3 in the dynamic approach. Thus, one of these assignments is possibly a false-positive. However, with a hR_F of 64 and 68, zones 3 and 4 are close together, and the peak width of methabenzthiazuron by separation with MP 2 amounted to 2.8 mm (hR_F = 64–68). Through an extraction width of 2 mm, it is quite possible that the substance actually occurs in both zones 3.2 and 4.3.

With the investigation of the spiked surface water, the advantages of 2D separations and especially of the dynamic approach were shown. Through adjusted MPs in the second dimension, much better separation was achieved, and more candidates could be matched to an effective zone. One example is zone 6, which could not be divided by MP B, but was separated to five effective zones with MP D. For two of the five effective zones, a matching candidate could also be assigned.

This developed concept now could be applied to complex environmental samples. When the effective compounds in the samples are completely unknown coupling to HRMS gets rudimentary for identification. Through the used elution head-based extraction a coupling to HRMS would be easily realizable, but first of all the recovery of twice done extraction of effective zones (from 1D to 2D, from 2D to HRMS) has to be determined. But through variable

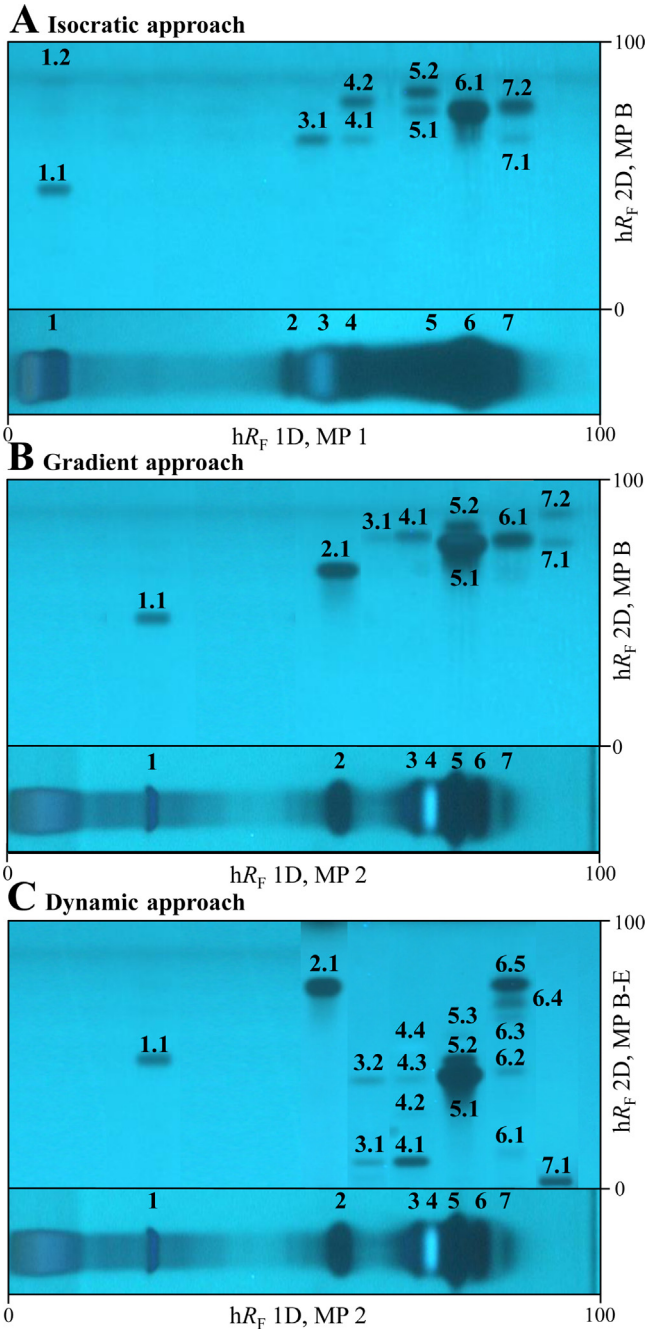


Fig. 5. Selective 2D HPTLC-AChE inhibition assay with spiked surface water. A: isocratic approach, B: gradient approach, C: dynamic approach.

application volume of HPTLC a possible loss during extraction could be equated.

4. Conclusions

In the current work, a strategy for selective 2D EDA with HPTLC as fractionation method was developed. Through application of elution head-based extraction, only effective zones were transferred from the first to the second dimension.

Three 2D approaches were developed and validated. The dynamic approach adjusting the MPs for the second dimension to the substanceís hR_F in the first dimension achieved the best results for peak capacity and orthogonality. Thus, peak capacity could be increased by the factor 6.8 compared to the 1D gradient development, and the potential of the method to compensate the lower peak capacity of HPTLC compared to 1D HPLC was confirmed. Peak capacities of several thousands, which were achieved with 2D HPLC cannot be reached with 2D HPTLC. But nevertheless HPTLC is a suitable fractionation method for EDA. The plate is solvent free after the separation, what tolerates the use of many different solvents and therefore leads to still passable orthogonality despite the same stationary phase. In 2D HPLC one challenge are incompatible solvents in the first and second dimension, but fractionation is more automated and therefore more suitable for high-throughput analysis.

A further increase in peak capacity may be achieved by the change in the stationary phase from instance normal to reversed phase. However, a challenge is the transfer of the bioassay from one to another stationary phase, because the pH and also the wettability of the plate have to be considered and so comparability of effects is not warranted [36].

By application of this method to spiked surface water, some neurotoxic effects could be clearly assigned to a spiked substance merely through determination of hR_F. Better peak capacity reduced the complexity of effective zones and thus facilitated the assignment of effective compounds. For the identification of unknown compounds, the coupling of this method to further analytical techniques such as MS or NMR would be the next important step. Hence, knowledge of the substanceís effects can be extended resulting in the assessment of their relevance and supporting the determination of limit values.

Acknowledgements

The authors thank the German Federal Ministry for Education and Research (BMBF) for the funding and the financial support of the project NeuroBox under the grant 02WRS1419E.

The authors gratefully acknowledge project students Robert Goßmann (Zweckverband Landeswasserversorgung, University of Applied Science, Aalen) and Andreas Taglieber (Zweckverband Landeswasserversorgung, University of Hohenheim, Stuttgart) for their assistance. We thank Tobias Bader and Wolfram Seitz (Zweckverband Landeswasserversorgung) for proof-reading and reviewing the article.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.chroma.2017.10.009.

References

[1] B.I. Escher, M. Allinson, R. Altenburger, P.A. Bain, P. Balaguer, W. Busch, J. Crago, N.D. Denslow, E. Dopp, K. Hilscherova, A.R. Humpage, A. Kumar, M. Grimaldi, B.S. Jayasinghe, B. Jarosova, A. Jia, S. Makarov, K.A. Maruya, A. Medvedev, A.C. Mehinto, J.E. Mendez, A. Poulsen, E. Prochazka, J. Richard, A. Schifferli, D. Schlenk, S. Scholz, F. Shiraishi, S. Snyder, G. Su, J.Y.M. Tang, B. v. d. Burg, S.C. v. d. Linden, S.D. Werner, C.K.C. Wong, M. Yang, B.H.Y. Yeung, X. Zhang, F.D.L. Leusch, Benchmarking organic micropollutants in wastewater, recycled water and drinking water with in vitro bioassays, Environ. Sci. Technol. 48 (2014) 1940.

[2] R.P. Schwarzenbach, B.I. Escher, K. Fenner, T.B. Hofstetter, C.A. Johnson, U. von Gunten, B. Wehrli, The challenge of micropollutants in aquatic systems, Science 313 (2006) 1072.

[3] M. König, B.I. Escher, P.A. Neale, M. Krauss, K. Hilscherová, J. Novák, I. Teodorović, T. Schulze, S. Seidensticker, M.A. Kamal Hashmi, J. Ahlheim, W. Brack, Impact of untreated wastewater on a major European river evaluated with a combination of in vitro bioassays and chemical analysis, Environ. Pollut. 220 (Part B) (2017) 1220.

[4] W. Brack, Effect-directed analysis: a promising tool for the identification of organic toxicants in complex mixtures? Anal. Bioanal. Chem. 377 (2003) 397.

[5] M.G. Weller, A unifying review of bioassay-guided fractionation effect-directed analysis and related techniques, Sensors 12 (2012) 9181.

[6] W. Brack, S. Ait-Aissa, R.M. Burgess, W. Busch, N. Creusot, C. Di Paolo, B.I. Escher, L. Mark Hewitt, K. Hilscherova, J. Hollender, H. Hollert, W. Jonker, J.

Kool, M. Lamoree, M. Muschket, S. Neumann, P. Rostkowski, C. Ruttkies, J. Schollee, E.L. Schymanski, T. Schulze, T.-B. Seiler, A.J. Tindall, G. De Aragão Umbuzeiro, B. Vrana, M. Krauss, Effect-directed analysis supporting monitoring of aquatic environments—an in-depth overview, Sci. Total Environ. 544 (2016) 1073.
[7] G. Morlock, W. Schwack, Hyphenations in planar chromatography, J. Chromatogr. A 1217 (2010) 6600.
[8] S.C. Weiss, N. Egetenmeyer, W. Schulz, in: G. Reifferscheid, S. Buchinger (Eds.), In Vitro Environmental Toxicology–Concepts, Application and Assessment, Springer Berlin, Heidelberg, 2016, p. 187.
[9] M. Á. Móricz, P.G. Ott, T.T. Häbe, A. Darcsi, A. Böszörményi, Á, D. Alberti Krüzselyi, P. Csontos, S. Béni, G.E. Morlock, Effect-directed discovery of bioactive compounds followed by highly targeted characterization, isolation and identification, exemplarily shown for solidago virgaurea, Anal. Chem. 88 (2016) 8202.
[10] Á.M. Móricz, T.T. Häbe, A. Böszörményi, P.G. Ott, G.E. Morlock, Tracking and identification of antibacterial components in the essential oil of Tanacetum vulgare L. by the combination of high-performance thin-layer chromatography with direct bioautography and mass spectrometry, J. Chromatogr. A 1422 (2015) 310.
[11] I. Yüce, G.E. Morlock, Streamlined structure elucidation of an unknown compound in a pigment formulation, J. Chromatogr. A 1469 (2016) 120.
[12] K. Burger, Thin layer chromatography with automated multiple development (AMD-TLC), Chem. Plant Prot. 12 (1995) 181.
[13] C. Stiefel, S. Dietzel, M. Endress, G.E. Morlock, Separation of pigment formulations by high-performance thin-layer chromatography with automated multiple development, J. Chromatogr. A 1462 (2016) 134.
[14] B. Milz, K. Karl-Friedrich, B. Spangenberg, Quantitative two-dimensional thin-layer chromatography using a diode-array detector, J. Planar Chromatogr. – Mod. TLC 25 (2012) 493.
[15] B. Spangenberg, Two-dimensional thin-layer chromatography of polyaromatic hydrocarbons measured by absorption, fluorescence, and chemiluminescence, J. Planar Chromatogr. – Mod. TLC 28 (2015) 139.
[16] T. Tuzimski, Some aspects of multidimensional chromatography techniques coupled with mass spectrometry applied for the separation of multicomponent mixtures of pesticides, J. Planar Chromatogr. – Mod. TLC 28 (2015) 190.
[17] X. Ouyang, P.E.G. Leonards, Z. Tousova, J. Slobodnik, J. de Boer, M.H. Lamoree, Rapid screening of acetylcholinesterase inhibitors by effect-directed analysis using LC×LC fractionation, a high throughput in vitro assay, and parallel identification by time of flight mass spectrometry, Anal. Chem. 88 (2016) 2353.
[18] G. Morlock, W. Schwack, Coupling of planar chromatography to mass spectrometry, TrAC–Trend Anal. Chem. 29 (2010) 1157.
[19] H. Luftmann, A simple device for the extraction of TLC spots: direct coupling with an electrospray mass spectrometer, Anal. Bioanal. Chem. 378 (2004) 964.
[20] C. Weins, H. Jork, Toxicological evaluation of harmful substances by in situ enzymatic and biological detection in high-performance thin-layer chromatography, J. Chromatogr. A 750 (1996) 403.
[21] A. Marston, Thin-layer chromatography with biological detection in phytochemistry, J. Chromatogr. A 1218 (2011) 2676.
[22] A. Marston, J. Kissling, K. Hostettmann, A rapid TLC bioautographic method for the detection of acetylcholinesterase and butyrylcholinesterase inhibitors in plants, Phytochem. Anal. 13 (2002) 51.
[23] T. Mroczek, Highly efficient, selective and sensitive molecular screening of acetylcholinesterase inhibitors of natural origin by solid-phase extraction-liquid chromatography/electrospray ionisation-octopole-orthogonal acceleration time-of-flight-mass spectrometry and novel thin-layer chromatography-based bioautography, J. Chromatogr. A 1216 (2009) 2519.
[24] R. Akkad, W. Schwack, Multi-enzyme inhibition assay for the detection of insecticidal organophosphates and carbamates by high-performance thin-layer chromatography applied to determine enzyme inhibition factors and residues in juice and water samples, J. Chromatogr. B 878 (2010) 1337.
[25] T. Bader, W. Schulz, K. Kümmerer, R. Winzenbacher, General strategies to increase the repeatability in non-target screening by liquid chromatography-high resolution mass spectrometry, Anal. Chim. Acta 935 (2016) 173.
[26] G.E. Morlock, Background mass signals in TLC/HPTLC–ESI–MS and practical advices for use of the TLC-MS interface, J. Liq. Chromatogr. Relat. Technol. 37 (2014) 2892.
[27] C. Weins, H. Jork, Toxicological evaluation of harmful substances by in situ enzymatic and biological detection in high-performance thin-layer chromatography, J. Chromatogr. A 750 (1996) 403.
[28] W. Schulz, W. Seitz, S.C. Weiss, W.H. Weber, M. Böhm, D. Flottmann, Use of Vibrio fischeri for screening for bioactivity in water analysis, J. Planar Chromatogr. 21 (2008) 427.
[29] M. Gilar, P. Olivova, A.E. Daly, J.C. Gebler, Orthogonality of separation in two-dimensional liquid chromatography, Anal. Chem. 77 (2005) 6426.
[30] F. Geiss, Fundamentals of Thin Layer Chromatography (Planar Chromatography), Dr. A. Hüthig Verlag, Heidelberg, 1987.
[31] L.R. Snyder, Classification off the solvent properties of common liquids, J. Chromatogr. Sci. 16 (1978) 223.
[32] M. Gilar, A.E. Daly, M. Kele, U.D. Neue, J.C. Gebler, Implications of column peak capacity on the separation of complex peptide mixtures in single- and two-dimensional high-performance liquid chromatography, J. Chromatogr. A 1061 (2004) 183.
[33] K. Horie, H. Kimura, T. Ikegami, A. Iwatsuka, N. Saad, O. Fiehn, N. Tanaka, Calculating optimal modulation periods to maximize the peak capacity in two-dimensional HPLC, Anal. Chem. 79 (2007) 3764.
[34] X. Li, D.R. Stoll, P.W. Carr, Equation for peak capacity estimation in two-dimensional liquid chromatography, Anal. Chem. 81 (2009) 845.
[35] D.R. Stoll, X. Li, X. Wang, P.W. Carr, S.E.G. Porter, S.C. Rutan, Fast, comprehensive two-dimensional liquid chromatography, J. Chromatogr. A 1168 (2007) 3.
[36] I.A. Ramallo, P. García, R.L.E. Furlan, A reversed-phase compatible thin-layer chromatography autography for the detection of acetylcholinesterase inhibitors, J. Sep. Sci. 38 (2015) 3788.

Selective two-dimensional effect-directed analysis with thin-layer chromatography

Supplementary Material

Lena Stütz[a,b*], Stefan C. Weiss[a], Wolfgang Schulz[a], Wolfgang Schwack[b], Rudi Winzenbacher[a]

[a]Laboratory for Operation Control and Research, Zweckverband Landeswasserversorgung, Am Spitzigen Berg 1, 89129 Langenau, Germany

[b]Institute of food chemistry, University of Hohenheim, Garbenstraße 28, 70599 Stuttgart, Germany

E-mail addresses:
Stuetz.L@lw-online.de
Weiss.S@lw-online.de
Schulz.W@lw-online.de
Wolfgang.Schwack@uni-hohenheim.de
Winzenbacher.R@lw-online.de

*Corresponding author:
Lena Stütz
E-mail address: Stuetz.L@lw-online.de
Tel.: +49 (7345) 9638-2226
Fax: +49 (7345) 9638-2290

Table of contents:

Table S1: Ingredients of the neurotoxic mix used for spiking of surface water and their retardation factors (hR_F) (substances which hR_F in the first dimension were less than ± 2 away from edge to another retardation area were measured twice with both possible mobile phases (MP) in the dynamic approach. The name of the used mobile phase for determining of the hR_F is given in subscript).

No.	Substance name	hR_F MP 1	hR_F MP 2	hR_F MP B	hR_F MP B - E
1	3-Hydroxycarbofuran	55	52	70	76_C
2	Acephate	24	29	54	54_B
3	Acridine	56	69	82	55_D
4	Aminocarb	58	66	78	30_D
5	Asulam	62	49	50	45_C
6	Azamethiphos	61	65	81	19_D
7	Azinphos-methyl	90	79	88	80_D / 1_E
8	Benfuracarb	82	71	80	46_D
9	Bromophos-ethyl	97	88	90	93_E
10	Caffeine	22	39	69	69_B / 10_C
11	Carbetamide	46	53	71	71_C
12	Carbosulfan	82	80	79	46_D / 3_E
13	Chlorbromuron	81	78	81	79_D
14	Chlorfenvinphos	82	72	86	63_D
15	Chloroxuron	62	62	75	57_C / 27_D
16	Chlorpyriphos	96	88	90	82_E
17	Diazinon	87	78	88	73_D
18	Dichlorophos	71	72	81	59_D
19	Dicrotophos	14	40	70	70_B / 4_C
20	Difenoxuron	55	56	75	49_C
21	Dimefuron	59	45	72	43_C
22	Dimethoate	53	52	72	32_C
23	Diuron	67	62	70	64_C / 33_D
24	Donepezil	2	22	56	56_B
25	Fenthion	95	86	90	42_E
26	Fluometuron	67	61	68	72_C / 32_D
27	Isoprocarb	82	74	77	60_D
28	Isoproturon	53	58	73	64_C
29	Linuron	84	77	81	76_D
30	Malathion	91	79	88	90_D / 0_E
31	Mephosfolan	49	50	77	25_C
32	Methabenzthiazuron	59	66	49	46_D
33	Methacriphos	59	81	87	28_D / 7_E
34	Methamidophos	22	34	47	2_B
35	Methidathion	89	80	88	87_D / 1_E
36	Methiocarb	84	75	80	70_D
37	Metobromuron	78	75	81	68_D
38	Metoxuron	51	56	70	48_C
39	Monolinuron	77	77	80	67_D
40	Paraoxon	73	73	85	46_D
41	Phosalone	93	84	89	6_E

No.	Substance name	hR_F MP 1	hR_F MP 2	hR_F MP B	hR_F MP B - E
42	Phosphamidon	45	54	78	32_C
43	Pirimicarb	64	66	83	22_D
44	Promecarb	87	77	80	70_D
45	Propetamphos	91	81	87	90_D / 2_E
46	Propham	85	81	82	89_D / 7_E
47	Rivastigmin	1	14	19	-
48	Triallate	93	86	89	46_E
49	Triazophos	88	78	86	65_D
50	Trichlorfon	70	76	80	51_D

Table S2: Overview of the used mobile phases

Dimension	Mobile phase	Composition	Steps	Migration distance [mm]
1D	MP 1	Formic acid-acetonitrile-dichloromethane (1:50:49, v/v/v)	1	70.0
	MP 2	Methanol (0.05 % v/v formic acid)-dichloromethane-n-hexane		
		(100:0:0, v/v/v)	1	11.0
		(100:0:0, v/v/v)	2	11.0
		(100:0:0, v/v/v)	3	11.0
		(70:30:0, v/v/v)	4	16.3
		(30:70:0, v/v/v)	5	21.6
		(20:80:0, v/v/v)	6	26.9
		(15:85:0, v/v/v)	7	32.2
		(11:89:0, v/v/v)	8	37.5
		(8.5:91.5:0, v/v/v)	9	42.8
		(6.5:93.5:0, v/v/v)	10	48.2
		(5:95:0, v/v/v)	11	53.5
		(4:96:0, v/v/v)	12	58.8
		(3.5:96.5:0, v/v/v)	13	64.1
		(0:100:0, v/v/v)	14	69.4
		(0:50:50, v/v/v)	15	74.7
		(0:0:100, v/v/v)	16	80.0
2D	MP A	Formic acid-methanol-chloroform (3.3:36.6:60, v/v/v)	1	70.0
	MP B	Methanol-chloroform (20:80, v/v)	1	70.0
	MP C	Ethyl acetate-dichloromethane-n-hexane (65:10:25, v/v/v)	2	70.0
	MP D	Acetonitrile-chloroform-n-hexane (7:70:23, v/v/v)	3	70.0
	MP E	Toluene-n-hexane (70:30, v/v)	2	70.0

Table S3: hR_F and normalized hR_F (feature scaling) of 25 substances in 1D (MP 1) and 2D (MP B) for investigation of orthogonality of the isocratic approach.

No.	Substance	hR_F 1D	hR_F 1D normalized	hR_F 2D	hR_F 2D normalized
1	4-Hydroxydiclofenac	53	56	43	46
2	Acephate	24	23	54	59
3	Aldicarb sulfoxide	14	11	55	60
4	Alprenolol	4	0	6	1
5	Benazolin	49	52	11	7
6	Bitertanol	49	52	71	80
7	Bupirimate	81	89	85	96
8	Carbendazim	32	32	69	77
9	Chloramphenicol	47	49	48	52
10	Ethidimuron	63	68	59	65
11	Ethirimol	8	5	60	66
12	Fenitrothion	91	100	88	100
13	Fenoxaprop	49	52	19	17
14	Foramsulfuron	24	23	58	64
15	Ketoprofen	48	51	18	16
16	Methacriphos	89	98	87	99
17	Monocrotophos	22	21	63	70
18	N-Acetyl sulfamethoxazole	54	57	54	59
19	Omethoate	19	17	60	66
20	Pentoxyfylline	27	26	66	73
21	Propazin	81	89	81	92
22	Pyroquilon	50	53	80	90
23	Rimsulfuron	61	66	66	73
24	Triclopyr	47	49	12	8
25	Valsartan acid	47	49	5	0
	Min	4		5	
	Max	91		88	

$$hR_{F,normalized} = (hR_F - hR_{F,min}) / (hR_{F,max} - hR_{F,min}) * 100$$

$$O_\perp = \left(\sum bins - \sqrt{P_{max}}\right) / (0.63 \cdot P_{max})$$

$$O_\perp = (10 - \sqrt{25}) / (0.63 \cdot 25) = 0.32$$

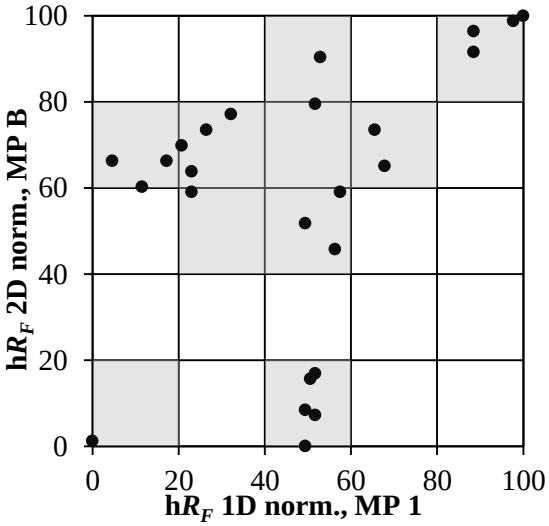


Figure S1: Illustration of occupied bins through 2D separation of 25 substances with the isocratic approach

Table S4: hR_F and normalized hR_F of 25 substances in 1D (MP 2) and 2D (MP B) for investigation of orthogonality of the gradient approach.

No.	Substance	hR_F 1D	hR_F 1D normalized	hR_F 2D	hR_F 2D normalized
1	4-Hydroxydiclofenac	32	21	43	46
2	Acephate	29	17	54	59
3	Aldicarb sulfoxide	28	15	55	60
4	Alprenolol	18	0	6	1
5	Benazolin	25	11	11	7
6	Bitertanol	37	29	71	80
7	Bupirimate	67	74	85	96
8	Carbendazim	38	30	69	77
9	Chloramphenicol	34	24	48	52
10	Ethidimuron	45	41	59	65
11	Ethirimol	26	12	60	66
12	Fenitrothion	84	100	88	100
13	Fenoxaprop	28	15	19	17
14	Foramsulfuron	25	11	58	64
15	Ketoprofen	28	15	18	16
16	Methacriphos	80	94	87	99
17	Monocrotophos	30	18	63	70
18	N-Acetyl sulfamethoxazole	30	18	54	59
19	Omethoate	31	20	60	66
20	Pentoxyfylline	36	27	66	73
21	Propazin	66	73	81	92
22	Pyroquilon	55	56	80	90
23	Rimsulfuron	28	15	66	73
24	Triclopyr	29	17	12	8
25	Valsartan acid	24	9	5	0
	Min	18		5	
	Max	84		88	

$$hR_{F,normalized} = (hR_F - hR_{F,min}) / (hR_{F,max} - hR_{F,min}) * 100$$

$$O_\perp = \left(\sum bins - \sqrt{P_{max}}\right) / (0.63 \cdot P_{max})$$

$$O_\perp = (9 - \sqrt{25}) / (0.63 \cdot 25) = 0.25$$

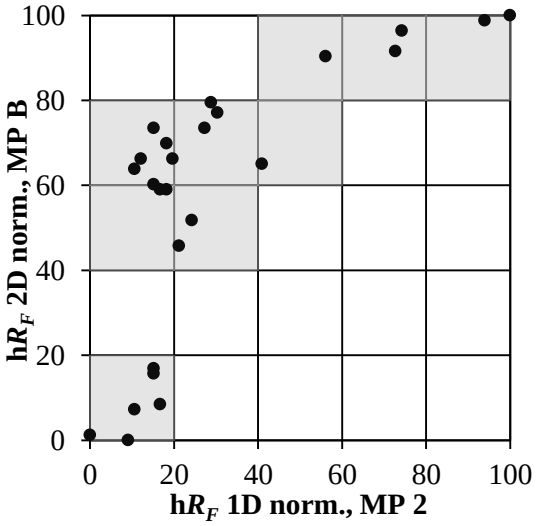


Figure S2: Illustration of occupied bins through 2D separation of 25 substances with the gradient approach

Table S5: hR_F and normalized hR_F of 125 substances in 1D (MP 2) and 2D (MP A - E) for investigation of orthogonality of the dynamic approach.

No.	**Substance**	**hR_F 1D**	**hR_F 1D normalized**	**hR_F 2D**	**hR_F 2D normalized**
1	1-(2-Chlorophenyl)-3-phenylurea	71	78	65	66
2	2,6-Dichlorbenzamide	55	60	85	87
3	2-Aminobenzothiazole	41	44	53	54
4	2-Hydroxybenzothiazole	59	65	93	96
5	3,4-Dichloranilin	78	86	61	62
6	4-Hydroxydiclofenac	32	34	43	43
7	Acephate	29	31	54	55
8	Acetamiprid	44	48	11	9
9	Aclonifen	85	94	42	42
10	Alachlor	75	83	76	78
11	Aldicarb sulfoxid	28	30	55	56
12	Ametryn	61	67	34	34
13	Amisulpride N-oxide	5	3	24	23
14	Atenolol	10	9	29	28
15	Azoxystrobin	63	69	15	14
16	Benazolin	25	26	11	9
17	Bezafibrate	26	27	31	31
18	Bisoprolol	17	17	68	69
19	Bitertanol	38	41	71	73
20	Boscalid	69	76	45	45
21	Bromacil	52	57	87	89
22	Bromophos ethyl	90	100	93	96
23	Bupirimate	67	74	42	42
24	Caffeine	39	42	69	71
25	Candesartan	24	25	12	11
26	Carbendazim	38	41	69	71
27	Carbosulfan	81	90	3	1
28	Cefixime	9	8	64	65
29	Chloramphenicol	34	36	48	48
30	Chlorotoluron	55	60	56	57
31	Ciprofloxacin	2	0	9	7
32	Clodinafop-propargyl	80	89	2	0
33	Clomazone	72	80	67	68
34	Clopyralid	18	18	67	68
35	Cyprodinil	70	77	57	58
36	Dapsone	50	55	46	46
37	Diazepam	52	57	70	72
38	Diazinon	72	80	73	75
39	Dichlofenthion	89	99	86	88
40	Diflubenzuron	74	82	59	60
41	Diflufenican	78	86	84	86
42	Dihydrocodeine	9	8	26	25

No.	Substance	hR_F 1D	hR_F 1D normalized	hR_F 2D	hR_F 2D normalized
43	Dimefuron	45	49	43	43
44	Dimethoat	52	57	32	32
45	Dimethomorph	45	49	15	14
46	Diphenylamine	87	97	72	74
47	Ditalimphos	75	83	50	51
48	Epoxiconazole	54	59	47	47
49	Estrone	65	72	41	41
50	Ethidimuron	44	48	21	20
51	Ethion	86	95	42	42
52	Ethirimol	26	27	60	61
53	Fenarimol	49	53	62	63
54	Fenitrothion	84	93	29	28
55	Fenobucarb	73	81	61	62
56	Fenoxaprop	28	30	19	18
57	Fenpropathrin	84	93	31	31
58	Fenpyroximate	67	74	54	55
59	Fenthion	86	95	42	42
60	Flecainide	15	15	61	62
61	Fluazinam	87	97	92	95
62	Fluazinofop	26	27	18	17
63	Flufenacet	72	80	54	55
64	Fluopicolide	75	83	72	74
65	Flurtamone	56	61	89	92
66	Foramsulfuron	25	26	58	59
67	Imazapyr	25	26	22	21
68	Iodipamid	13	13	53	54
69	Iodofenphos	89	99	86	88
70	Iotalamic acid	15	15	44	44
71	Iprovalicarb	58	64	96	99
72	Isoprocarb	73	81	60	61
73	Ketoprofen	28	30	18	17
74	Ketotifen	15	15	35	35
75	Kresoxim methyl	81	90	2	0
76	Melamin	12	11	20	19
77	Metamitron	41	44	24	23
78	Metformin	5	3	6	4
79	Methacriphos	80	89	7	5
80	Methidathion	80	89	87	89
81	Methiocarb	74	82	68	69
82	Metoprolol acid	11	10	50	51
83	Metsulfuron methyl	34	36	72	74
84	Monocrotophos	30	32	63	64
85	Myclobutanil	45	49	25	24
86	N-Acteyl sulfamethoxazol	34	36	54	55

No.	Substance	hR_F 1D	hR_F 1D normalized	hR_F 2D	hR_F 2D normalized
87	Nadolol	13	13	43	43
88	Naphazolin	10	9	42	42
89	Norflurazon	56	61	58	59
90	Olmesartan	18	18	68	69
91	Omethoate	31	33	60	61
92	Oxadixyl	52	57	51	52
93	Pencycuron	75	83	71	73
94	Pendimethalin	88	98	82	84
95	Pentoxifylline	36	39	66	67
96	Phenacetin	45	49	46	46
97	Phentoate	83	92	12	11
98	Phosalone	82	91	8	6
99	Phoxime	86	95	44	44
100	Picloram	17	17	61	62
101	Pirimiphos ethyl	81	90	6	4
102	Pirimiphos methyl	81	90	6	4
103	Procymidone	81	90	8	6
104	Profenophos	75	83	80	82
105	Profluralin	90	100	97	100
106	Propazine	66	73	42	42
107	Prosulfocarb	80	89	8	6
108	Pyroquilon	54	59	42	42
109	Rimsulfuron	28	30	66	67
110	Ritalinic acid	7	6	30	29
111	Roxithromycin	10	9	46	46
112	Sebuthylazine	63	69	35	35
113	Sitagliptin	10	9	26	25
114	Sulbactam	18	18	55	56
115	Sulfadimidin	41	44	51	52
116	Sulfamethoxazol	44	48	74	76
117	Sulpiride N-oxid	5	3	16	15
118	TCMTB	80	89	9	7
119	Thiourea	29	31	34	34
120	Tramadol	13	13	40	40
121	Triallate	84	93	46	46
122	Triclopyr	29	31	12	11
123	Triethylphosphate	41	44	19	18
124	Trimethoprim	15	15	49	49
125	Valsartan acid	24	25	5	3
	Min	2		2	
	Max	90		97	

$hR_{F,normalized} = (hR_F - hR_{F,min}) / (hR_{F,max} - hR_{F,min})$ *100

$O_\perp = \left(\sum bins - \sqrt{P_{max}}\right) / (0.63 \cdot P_{max})$

$O_\perp = (64 - \sqrt{125}) / (0.63 \cdot 11) = 0.65$

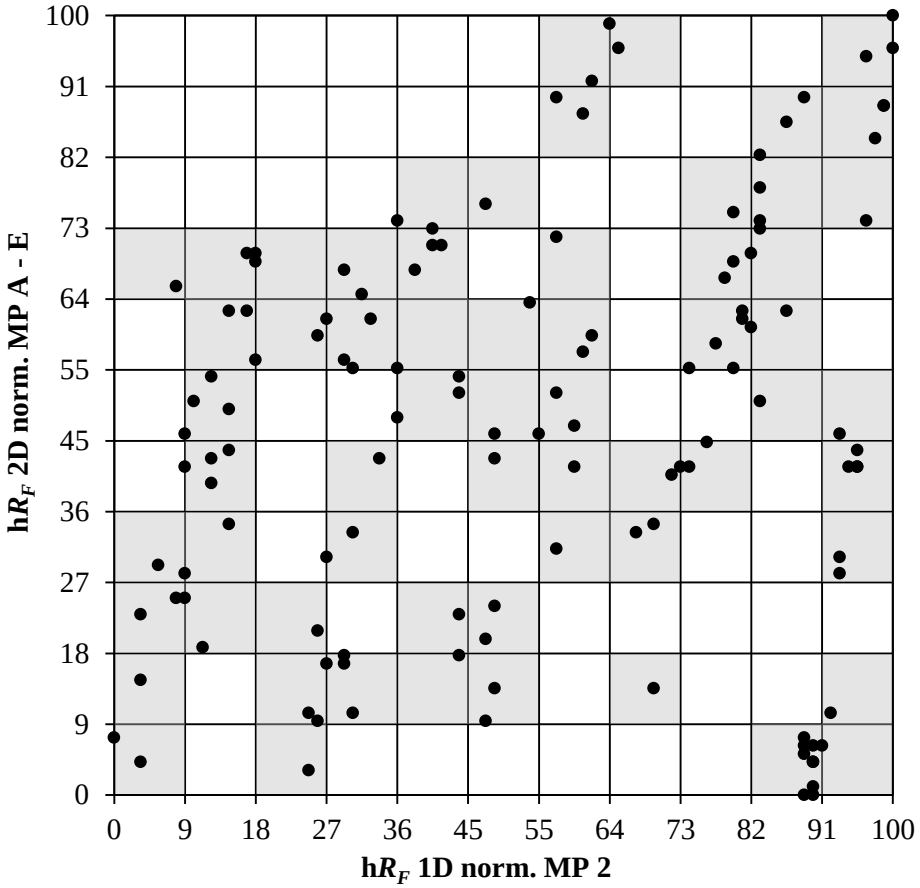


Figure 3: Illustration of occupied bins through 2D separation of 125 substances with the dynamic approach.

Publication 2

L. Stütz, P. Leitner, W. Schulz, R. Winzenbacher, Identification of genotoxic transformation products by effect-directed analysis with high-performance thin-layer chromatography and non-target screening, *Journal of Planar Chromatography - Modern TLC* 32 (2019) 3, 173-182, https://doi.org/10.1556/1006.2019.32.3.1.

JPC Original Research Papers

Identification of Genotoxic Transformation Products by Effect-Directed Analysis with High-Performance Thin-Layer Chromatography and Non-Target Screening

Lena Stütz*, Patricia Leitner, Wolfgang Schulz, and **Rudi Winzenbacher**

Key Words:

Metformin
Umu assay
Thin-layer chromatography–mass spectrometry Interface 2
Chlorination
Liquid chromatography
High-resolution mass spectrometry

Summary

The widespread use of the pharmaceutical metformin for diabetes therapy has led to finding its way into many surface waters up to the µg L^{-1} range and subsequently into different water treatment processes. In this study, metformin was treated with hypochlorite on the laboratory scale, and the resulting transformation products were investigated with the umu assay in a microtiter plate, where a genotoxic effect was detected. For the characterization of this genotoxic effect, the sample was separated using high-performance thin-layer chromatography (HPTLC), and 29 zones over the whole retardation area were extracted from the HPTLC plate with the thin-layer chromatography–mass spectrometry (TLC–MS) Interface. Then, the umu assay was performed again with each extracted zone, such that the genotoxic effect in the sample could be assigned to a certain zone. By the measurement of this effective zone with high-performance liquid chromatography with high-resolution mass spectrometry and by performing a non-target screening, the effective substance could be identified as a cyclic dehydro-1,2,4-triazole derivate with an intense yellow color. This substance formerly was found by *Armbruster et al.* (Water Research, 2015), which is a major transformation product of chlorine-treated metformin.

1 Introduction

Through human influences, increasing quantities of anthropogenic trace substances are found in the water cycle. Sophisticated methods must be used to remove these substances during drinking water treatment, using different processes, such as chlorination or ozonation in combination with filtration or activated carbon [1, 2]. While these oxidation processes disinfect water and prevent microbial contamination in drinking water [3], they also cause the degradation of organic substances into transformation products (TPs), resulting in a reduced toxicity in general [4–6].

Though many of these TPs are not characterized, and their impacts on humans and the ecosystem are not known, it is difficult to select the relevant ones, namely, those with toxicological effects. One opportunity for selection is effect-directed analysis (EDA), which is a combination of physicochemical fractionation, biotesting, and chemical analysis [7]. Fractionation makes it possible to assign individual fractions of a sample to the effects. This reduces the complexity of the substances contained in the whole sample to the substances present in the effective fraction [8].

A substantial endpoint, which is also crucial for the determination of limit values, is the genotoxicity. Substances with a demonstrated genotoxic effect are only allowed to occur in drinking water at levels ≤ 0.1 µg L^{-1} according to the health-related indicator value concept [9]. Different *in vitro* bioassays, such as the Ames fluctuation assay [10] or the micronucleus assay [11], can measure genotoxicity, and they complement each other. In our study, the *in vitro* umu assay according to DIN 38415-T3 (DIN: German Institute for Standardization) was used [12]. When genotoxic substances cause DNA damage in genetically modified *Salmonella typhimurium* TA1535/pSK1002, the SOS response starts, which triggers the genetic expression of β-galactosidase. The activity of β-galactosidase is determined through the addition of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) substrate, which is cleaved by β-galactosidase to the yellow *o*-nitrophenol (λ = 420 nm) [13]. Many substances achieve their genotoxic effects only after their activation in the xenobiotic metabolism of the liver, which is why the bioassay was performed with (+S9) and without (−S9) metabolic activation. For the execution of the metabolic activation in a microtiter plate, an enzyme mix obtained from rat livers was used. With the assays in the microtiter plate, the effects are detected as sum effects, since no fractionation of the samples is performed. This approach is advantageous,

L. Stütz, P. Leitner, W. Schulz, and R. Winzenbacher; Laboratory for Operation Control and Research, Zweckverband Landeswasserversorgung, Am Spitzigen Berg 1, 89129 Langenau, Germany; L. Stütz, Institute of Food Chemistry, University of Hohenheim, Garbenstraße 28, 70599 Stuttgart, Germany; P. Leitner, Faculty of Chemistry, Aalen University of Applied Sciences, Beethovenstraße 1, 73430 Aalen, Germany.
E-mail addresses: Stuetz.L@lw-online.de (*corresponding author);
Schulz.W@lw-online.de;
Winzenbacher.R@lw-online.de

since it reflects the real conditions in the environment, where individuals come into contact with the whole sample, but it hampers the identification of effective compounds.

For identification of effective compounds in complex samples, EDA is a suitable tool. Through fractionation, the complexity of the samples can be reduced, and the occurring effects can be matched to the related fraction. Different EDA approaches with high-performance liquid chromatography (HPLC) as the separation method are already known [14, 15]. High-performance thin-layer chromatography (HPTLC) has also been demonstrated to be a suitable separation technique for EDA [16, 17]. Rather insensitive bioassays can also be employed by applying high sample volumes to the HPTLC plate [18]. The separation with HPTLC is notably tolerant against matrix influences, and in contrast to LC, the ingredients of the sample, which cannot be separated and therefore remain on the application zone, can also be detected. Compared to LC separations, HPTLC has lower separation efficiency. The HPTLC gradient development was already shown to be a good strategy for improvement of peak capacity [19, 20]. Moreover, there are already multidimensional approaches that have been able to significantly increase the peak capacity of HPTLC [21]. In many cases, bioassays were applied directly on the HPTLC plate after separation [22]. However, the metabolic activation of the indirect genotoxic substances is currently not feasible using the HPTLC plate [23], which is why in this study, the assay was performed using a microtiter plate. With the so-called thin-layer chromatography–mass spectrometry (TLC–MS) Interface 2, zones can be extracted from the HPTLC plate and transferred to the bioassay in a microtiter plate and/or to the LC with high-resolution mass spectrometry (HRMS) [24]. A challenge of this technique is the lack of automation of the extraction with the TLC–MS Interface 2, so that manual effort is required. For the extraction from the HPTLC plate, the selection of zones is important. One opportunity is to extract zones over the whole retardation area in order to achieve a preferable comprehensive mapping of the effects in the bioassay or the signals (from now on called features) using HRMS. Another possibility is the selection of the extraction zones according to certain properties, such as fluorescence or absorption under 254 nm. Often, previous knowledge of the samples is available, which facilitates decision-making regarding where to extract the zones.

In this study, pharmaceutical metformin (MF), which has widespread uses in diabetes therapy, was investigated with the umu assay after treatment with hypochlorite. MF is commonly found in sewage and surface waters and is among the most produced pharmaceuticals worldwide. In the investigations of 5 German rivers, MF was detected from 1.3 up to 17 µg L^{-1}, while in the influent of a sewage treatment plant, concentrations of up to 129 µg L^{-1} were measured [25]. Since a genotoxic effect of the chlorinated MF sample in the umu assay could be detected, the aim of this study was to assign the effect to a transformation product of metformin. Therefore, the bioassay in the microtiter plate was coupled to HPTLC separation, and through the extraction of the effective zone with the TLC–MS Interface 2 and by performing a non-target screening, the effective compound could be identified. The structure of the identified yellow transformation product was elucidated in Ref. [26], but this study was able to assign a genotoxic effect to it and thus significantly increased its relevance.

2 Experimental

2.1 Chemicals and Reagents

Ultrapure water was acquired by using ultrapure apparatus (PURELAB Ultra, Elga LabWater, Lane End, United Kingdom). Acetonitrile (ROTISOLV ≥ 99.95%, LC–MS-grade) and 2-propanol (ROTISOLV ≥ 99.95%, LC–MS-grade) were purchased from Carl Roth (Karlsruhe, Germany). Dichloromethane and *n*-hexane were purchased from J.T. Baker (Center Valley, PA, USA). Methanol (AMD Chromasolv ≥ 99.9%) was supplied by Sigma-Aldrich (Steinheim am Albuch, Germany) and formic acid (98–100%) was supplied by Merck (Darmstadt, Germany).

Sodium hypochlorite (13% Cl_2) was purchased from Bernd Kraft (Duisburg, Germany), and sodium thiosulfate solution (1 mol L^{-1}) was supplied by Merck.

Salmonella typhimurium TA 1535/pSK1002 was obtained by the DSMZ (Leipzig, Germany). S9 Sprague Dawley (SD) rat liver extract was supplied by Trinova (Giessen, Germany). Cofactor β-nicotinamide-adenine-dinucleotide-phosphate (NADP) came from Carbolution (St. Ingbert, Germany) and glucose-6-phosphate (G6P) was supplied by AppliChem (Darmstadt, Germany). *O*-nitrophenyl-β-D-galactopyranoside (ONPG) was supplied by Merck. Bacto tryptone, potassium chloride, magnesium chloride hexahydrate, disodium hydrogen phosphate, sodium dihydrogen phosphate dihydrate, 2-mercaptoethanol, and disodium carbonate were obtained from Merck. Sodium chloride, ampicillin sodium salt, and sodium dodecyl sulfate were supplied by Sigma-Aldrich. HEPES and α-D-glucose were purchased from AppliChem.

2.2 Standards

Standard stock solutions were prepared by dissolving 10 mg of each pure substance in 100 mL of methanol and storing it at −18°C. To prepare the respective standard solutions, the stock standards were diluted in methanol to the required concentration. The standard solutions were used for 4 weeks.

The stability of HPTLC was examined with a mix consisting of 9 substances: 1,3,6-naphthalenesulfonic acid trisodium salt hydrate (Sigma-Aldrich, purity ≥ 90%), 1,5-naphthalenedisulfonic acid tetrahydrate (Sigma-Aldrich, purity ≥ 97%), 1-naphthalenesulfonic acid (Alfa Aesar, Haverhill, MA, USA, purity ≥ 99%), theobromine (Sigma-Aldrich, purity ≥ 98%), caffeine (Sigma-Aldrich, purity ≥ 99%), thiourea (Sigma-Aldrich, purity ≥ 99%), *N*-phenylacetamide (LGC-Standards, Teddington, United Kingdom, purity 100%), *N*-phenylbenzamide (Sigma-Aldrich, purity ≥ 98%), and *N*,*N*-dimethyl-4-[(E)-phenyldiazenyl]-aniline (Merck, purity 100%), with 300 ng on the HPTLC plate for each substance. The stability of the umu assay was reviewed with the two positive controls, namely, 4-nitroquinoline-*N*-oxide (4-NQO, −S9 activation to measure the direct genotoxicity) from Sigma-Aldrich (purity ≥ 98%) and 2-aminoanthracene (2-AA, +S9 activation to measure the indirect genotoxicity) from Alfa Aesar (purity ≥ 94%).

The metformin hydrochloride standard substance was supplied by Sigma-Aldrich (purity ≥ 99.2%). The yellow and colorless TPs were supplied by Technologiezentrum Wasser (Karlsruhe, Germany).

2.3 Treatment of Metformin with Chlorine

In a laboratory trial, 100 mg L^{-1} of metformin (MF, 0.77 mmol L^{-1}) in ultrapure water was treated with 2 (1.5 mmol L^{-1}), 4 (3.1 mmol L^{-1}), and 6 (4.6 mmol L^{-1}) molar equivalents (eq) of sodium hypochlorite, respectively. The active chlorine was determined iodometrically before each trial [27]. Because of the chlorate formation, the sodium hypochlorite solution was used for 6 weeks after procurement, only. After 60 min of stirring, the reaction was stopped by adding sodium thiosulfate. The dosage of thiosulfate was calculated, depending on the sodium hypochlorite concentration with a security factor of 1.4. In each trial, a blank without MF and a control with MF but without hypochlorite were established in parallel.

2.4 *Salmonella typhimurium* TA1535/pSK1002 Assay (Umu Assay)

The umu assay was performed according to the DIN 38415-T3. Briefly, *Salmonella typhimurium* TA1535/pSK1002 was cultivated and stored at −80°C in a ULUF 125 freezer from Arctiko (Esbjerg, Denmark). On the day of the execution of the assay, the overnight culture of bacteria was diluted at a ratio of 1:10 with fresh tryptone, glucose, and ampicillin (TGA) media and then was incubated again for 90 min at 37°C until an optical density (OD) of 350 formazine attenuation units (FAU, photometer Nanocolor 400D, Macherey Nagel, Düren, Germany) was reached. The microtiter plate was filled with 180 µL of the sample (different dilution levels), 20 µL of the fresh 10-fold concentrated TGA media, and 70 µL of inoculum. With metabolic activation also, 180 µL of the sample at different dilution levels, but 20 µL of the 10-fold concentrated TGA media with cofactors and 70 µL of the inoculum with the S9 liver extract were applied to the microtiter plate. The samples were investigated in triplicate and at six dilution levels (1:1.5, 1:3, 1:6, 1:12, 1:24, and 1:48) in ultrapure water. The incubation of the 96-well plate was done using a PHMP-4 Thermo Shaker (Grant Instruments, Shepreth, United Kingdom) for 2 h at 37°C. Then, the plate was diluted 10 times in fresh TGA media and incubated for another 2 h. The bacterial growth rates (GRs) were measured using a FLUOstar® Omega microtiter plate reader from BMG LABTECH (Ortenberg, Germany) at a wavelength of 600 nm. *O*-nitrophenyl-β-D-galactopyranoside (ONPG) was used as the substrate for the detection of the β-galactosidase activity. If genotoxic substances occurred in the sample, β-galactosidase was expressed and ONPG was cleaved to *o*-nitrophenol, which could be measured at 420 nm due to its yellow staining.

In the umu assay, the bacterial growth rate (GR) was calculated using the following formula (OD: optical density):

$$GR = \frac{sample\ OD_{600} - blank\ OD_{600}}{negative\ control\ OD_{600} - blank\ OD_{600}} \quad (1)$$

GRs smaller than 0.5 indicate cytotoxicity; thus, those wells of the microtiter plate could not be evaluated. The induction rate (IR) determined the genotoxic effect according to the following formula:

$$IR = \frac{1}{GR} * \frac{sample\ OD_{420} - blank\ OD_{420}}{negative\ control\ OD_{420} - blank\ OD_{420}} \quad (2)$$

In accordance with DIN 38415-T3, samples with an IR ≥ 1.5 were considered to be genotoxic. In the case of different IRs with and without metabolic activation, the larger IR was used. For the positive control substances, IR ≥ 2 was required. 4-Nitroquinoline-*N*-oxide was used as a positive control without metabolic activation. It achieved a mean IR of 3.69 (N = 16, standard deviation (SD) = 0.55). 2-Aminoanthracene was applied as a positive control with metabolic activation, and a mean IR of 3.47 (N = 15, SD = 0.63) was determined.

The results of the assays were indicated as the lowest ineffective dilution (LID). The LID is the lowest dilution level within the assay that does not show any genotoxic effect (IR < 1.5). Thus, higher LID values mean higher genotoxicity.

2.5 High-Performance Thin-Layer Chromatography (HPTLC)

2.5.1 HPTLC Apparatus and Software

For the application, separation and detection of HPTLC plates, an automatic TLC sampler 4 (ATS 4), an automated multiple development 2 (AMD 2), a TLC Scanner 3, and a TLC visualizer 2 were used. The associated software was winCATS (1.4.9). The elution of the zones from the plate was performed using the TLC–MS Interface 2 with an oval elution head (4 mm × 2 mm). All devices were from CAMAG (Muttenz, Switzerland).

2.5.2 High-Performance Thin-Layer Chromatography

Before they were used, the HPTLC LiChrospher silica gel 60 F_{254S} plates (Merck) were immersed twice in 2-propanol for

Table 1

Composition of the gradient development with AMD 2.

Step	Methanol–formic acid 100:0.05 (*V*/*V*) (%)	Dichloromethane [%]	*n*-Hexane [%]	Migration distance [mm]
1	100.0	0.0	0.0	11.0
2	100.0	0.0	0.0	11.0
3	100.0	0.0	0.0	11.0
4	70.0	30.0	0.0	16.3
5	30.0	70.0	0.0	21.6
6	20.0	80.0	0.0	26.9
7	15.0	85.0	0.0	32.2
8	11.0	89.0	0.0	37.5
9	8.5	91.5	0.0	42.8
10	6.5	93.5	0.0	48.2
11	5.0	95.0	0.0	53.5
12	4.0	96.0	0.0	58.8
13	3.5	96.5	0.0	64.1
14	0.0	100.0	0.0	69.4
15	0.0	50.0	50.0	74.7
16	0.0	0.0	100.0	80.0

20 min. After drying at 120°C using a TLC plate heater (approximately 20 min, CAMAG), the plates were pre-developed to the top edge with acetonitrile. Finally, the plates were heated again to 120°C for 20 min. The cleaned plates were stored in a desiccator at room temperature.

The samples were applied to the HPTLC plate in a 6 mm × 3 mm area with 500 µL each. For separation, the AMD 2 was used. The plate was developed in a stepwise manner with increasing migration distances. Each step consisted of another solvent composition with decreasing eluent strength. The first three separation steps were necessary to focus the substances on the application area (y = 6.5 − 9.5 mm) to a thin band **(Table 1).**

The stability of chromatography was examined using a mix consisting of nine substances, which were distributed over the whole retardation area after separation: 1,3,6-naphthalenesulfonic acid (100-fold retardation factor [hR_F] = 12, % relative standard deviation [*%RSD*] = 6%), 1,5-naphthalenesulfonic acid (hR_F = 14, *%RSD* = 6%), 1-naphthalenesulfonic acid (hR_F = 20, *%RSD* = 5%), thiourea (hR_F = 28, *%RSD* = 6%), theobromine (hR_F = 31, *%RSD* = 8%), caffeine (hR_F = 41, *%RSD* = 8%), *N*-phenylacetamide (hR_F = 56, *%RSD* = 4%), *N*-phenylbenzamide (hR_F = 77, *%RSD* = 2%), and *N*,*N*-dimethyl-4-[(*E*)-phenyldiazenyl]-aniline (hR_F = 83, *%RSD* = 1%). There were N = 42 HPTLC plates over a period of 6 months.

2.5.3 HPTLC Extraction with the TLC–MS Interface 2

For the extraction of the zones from the plate, the TLC–MS Interface 2 was used, where the elution head was pressed to the HPTLC plate for 1 min with a flow of 200 µL min^{-1}. For the extraction of the zones, ultrapure water was used as eluent, because the bacteria do not tolerate organic solvents, and for the normal phase HPTLC plates, water has high eluent strength. In order to obtain sufficient extracted volume for the umu assay (different dilution levels, measurements in triplicate, parallel approach with and without metabolic activation, altogether 2160 µL extract volume required), the separation of the sample and the extraction of the effective zones have to be repeated 12 times. This is why one sample was applied several times to one plate. Subsequently, the extracts were combined in one vial.

To avoid a carryover, after each extraction, the elution head was pressed to an empty site of the HPTLC plate and was purged with ultrapure water for at least 3 min. For the LC–HRMS measurement, additional to the effective zone, a blank from the HPTLC plate was always required. First investigations showed that the blanks within one plate are quite different. Therefore, the blank extraction was done on an empty track directly beside the effective zone with the same hR_F. In the non-target evaluation, a feature was considered valid if its signal was at least five times higher than the corresponding signal in the blank. This allowed false positive results to be ruled out, which were caused by blank values from the plate material or the binder.

2.6 Non-Target Screening with LC–HRMS

LC–HRMS analysis was performed with the Shimadzu Nexera Prominence LC (Kyoto, Japan) coupled to a quadrupole-time-of-flight–mass spectrometer (QTOF–MS X500R, Sciex, Framingham, MA, USA). Each sample (injection volume: 10 µL) was analyzed in triplicate using reversed-phase separation (Zorbax Eclipse Plus C18, 2.1 mm × 150 mm, 3.5 µm, Agilent, Santa Clara, CA, USA) with a flow of 0.3 mL min^{-1}. An LC gradient, consisting of (A) water (0.1%, *V*/*V* formic acid) and (B) acetonitrile (0.1%, *V*/*V* formic acid) with the following parameters, was used: 2% B (0 min), 2% B (1 min), 20% B (2 min), 100% B (16.5 min), 100% B (22 min), 2% B (22.1 min), and 2% B (33 min). For ionization, electrospray ionization (ESI) was used in the positive mode, and ions were monitored within the mass range of m/z 100 up to 1200. The acquisition of the MS^2 spectra was performed using data dependent acquisition (DDA) experiments for a maximum of 12 precursors with the highest intensity (after dynamic background subtraction) within one cycle. After fragmentation by collision-induced dissociation (CID) with collision energy ramped from 20 to 55 V, fragment ions were acquired between m/z 30 and 1200.

The data acquisition was done *via* the Sciex OS (1.2) software. For non-target screening, the MarkerView (1.2.1) software was used for finding and aligning the peaks across different samples and replicates, and the Sciex OS (1.2) software (Analytics tool) was required for the peak integration. All further processing steps were performed with in-house MATLAB scripts (R2017a, The MathWorks, Inc., Natick, MA, USA). The detailed procedure of the used non-target evaluation is described in Ref. [28].

3 Results and Discussion

3.1 Investigations on the Genotoxicity of Chlorinated Metformin Samples

After a 5-min reaction time with hypochlorite, the chlorinated MF samples (treated with 2, 4, and 6 eq NaOCl) turned to an intense yellow color, where the sample MF with 4 eq NaOCl (MF 4, analogue abbreviations: MF 2 and MF 6) displayed the yellow staining with the highest intensity. The three samples,

Table 2

Maximal IR and LID of the umu assay from the investigation of chlorinated MF samples. The respective dilution level of the maximal IR is listed in the brackets.

Sample[a]	−S9 activation		+S9 activation	
	Max. IR[b] (dilution level)	LID	Max. IR[b] (dilution level)	LID
MF 2[c]	2.94 (3)	6	1.84 (1.5)	3
MF 4[d]	2.96 (6)	24	4.92 (1.5)	12
MF 6[e]	2.46 (3)	6	3.08 (1.5)	6
Blank[f]	0.95 (12)	1.5	1.28 (24)	1.5
Control[g]	1.21 (6)	1.5	1.15 (6)	1.5

[a] Direct analysis of the samples after chlorination without HPTLC separation
[b] Criterion for genotoxicity: IR ≥ 1.5
[c] MF with 2 eq hypochlorite
[d] MF with 4 eq hypochlorite
[e] MF with 6 eq hypochlorite
[f] Ultrapure water with hypochlorite
[g] MF in ultrapure water without hypochlorite

the blank (ultrapure water with hypochlorite), and the control (MF in ultrapure water without hypochlorite) were investigated using the umu assay with and without metabolic activation **(Table 2).**

All three samples showed a genotoxic effect in the umu assay. The highest IR of 4.92 was measured for sample MF 4 with metabolic activation. Therefore, the assay was repeated once more with this sample, and the effect could be confirmed. Moreover, it was observed that the lowest dilution level (1.5) of all three samples and the dilution level 3 of sample MF 4 without metabolic activation were cytotoxic to the bacteria. The GRs of these attempts were below 0.5, which is why the IR must not be calculated. The blank and the control did not show any genotoxic effect, which is why the highest LID of 1.5 was assigned.

For the confirmation of the potential genotoxic effect of the sample, different bioassays with the endpoint genotoxicity should be performed. Therefore, the AMES fluctuation assay (DIN 38415-4) and the micronucleus assay (DIN EN ISO 21427-2) were chosen. The sample MF 4, the blank, and the control were investigated with the assays, and the genotoxic effect of the sample MF 4 could be confirmed with the AMES assay. *Salmonella typhimurium* strain TA100, which reflects point mutations, showed a dose-dependent genotoxic effect with and without metabolic activation. The highest IR of 4.9 was detected with metabolic activation at the smallest dilution level of 3. With the bacteria strain TA98, in the detection of frameshift mutations, no genotoxic effect was measured. In the micronucleus assay that was performed with the Hep G2-cell line, the sample MF 4 only showed cytotoxic and secondary effects.

3.2 Workflow of EDA with HPTLC and Non-Target Screening

For the characterization of the measured genotoxic effects, an effect-directed analysis with the fractionation of the sample, the subsequent umu bioassay, and chemical detection was performed **(Figure 1).** Therefore, the sample MF 4 was applied to a HPTLC plate, separated *via* gradient development, and the zones were extracted with the TLC–MS Interface 2. The extracted zones were investigated with the umu assay to detect fractions with a genotoxic effect. The effective zone was further investigated with LC–HRMS, and a non-target screening was performed. The aim of this procedure was the identification of the genotoxic substance in the sample.

Instead of HPTLC, LC could also be used as a fractionation method, as has already been shown in some studies [14, 15]. This would have the advantage of a higher peak capacity of LC compared to HPTLC [7] as well as a better automation capability by direct spotting after separation into the microtiter plate. However, the solvent of the LC separation must be carefully evaporated before carrying out the bioassay. When using HPTLC, this step can be bypassed by aqueous extraction from the plate. Further advantages in favor of HPTLC as a fractionation technique are the already mentioned, namely, higher matrix tolerance compared to LC and the high variability of the application volume.

3.2.1 Coupling of HPTLC with the Umu Assay

The sample MF 4 was applied twice to the HPTLC plate, and gradient separation (composition, Table 1) was performed. Then, 29 zones were extracted with the TLC–MS Interface 2 over the whole retardation area, where extraction started at 10 mm up to 80 mm at an interval of 2.5 mm **(Figure 2B).** Since the elution head width is 2 mm, the extraction of 29 zones in one track would be too tight causing leakage of the elution head. Therefore, the extraction was performed by alternating between two tracks.

All 29 zones were extracted two times through the repeated application of the sample MF 4 on one HPTLC plate such that 400 µL of the extract volume per zone was available for the umu assay. Because of this limited available extract volume, the umu assay was performed without repetition and dilution but with and without metabolic activation, such that only 360 µL of extract per zone was required. With the extraction of 29 zones and an elution head width of only 2 mm, a distance of 0.5 mm (hR_F-range = 0.7) between the two zones was not extracted. Nevertheless, because of the substance's middle zone width of 2 mm [21] from the separation with the used gradient development, all of the zones were definitely covered but with recovery losses.

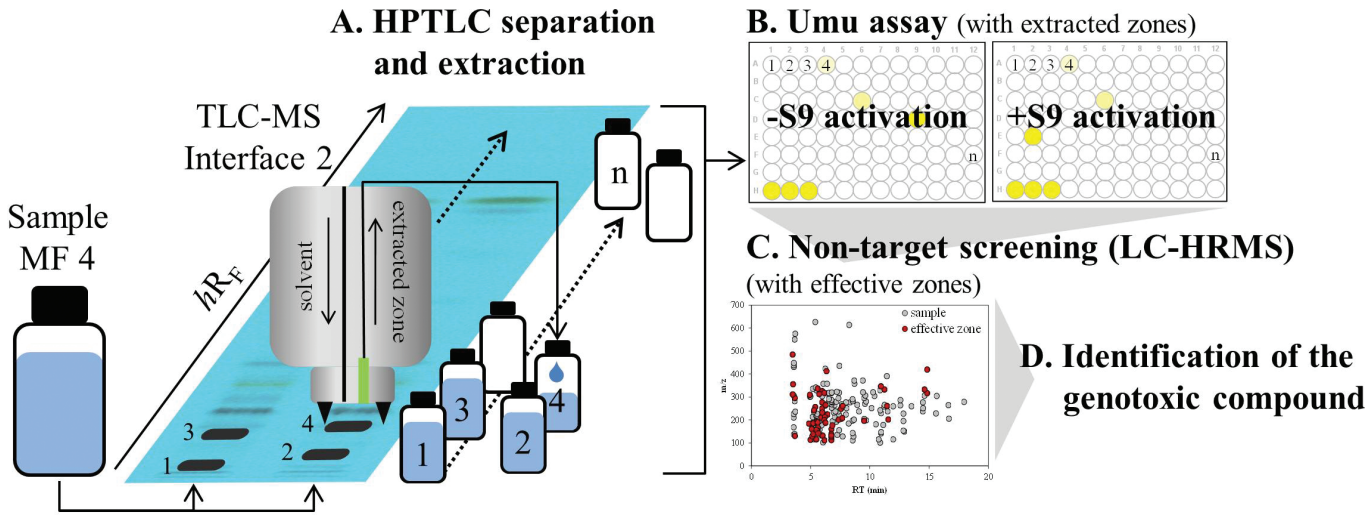


Figure 1

General workflow of EDA with HPTLC and non-target screening.

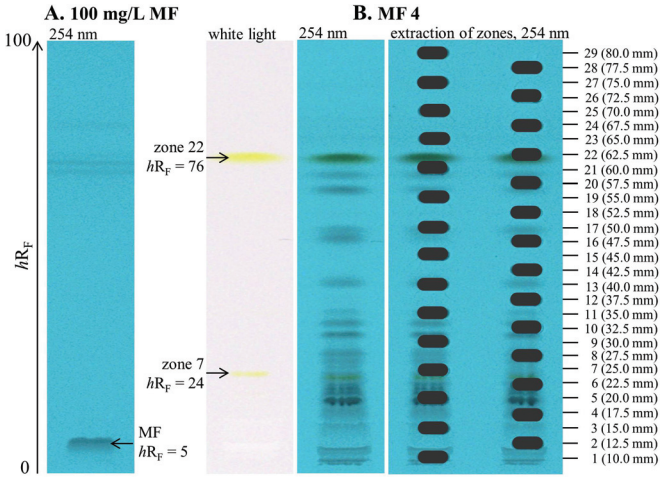


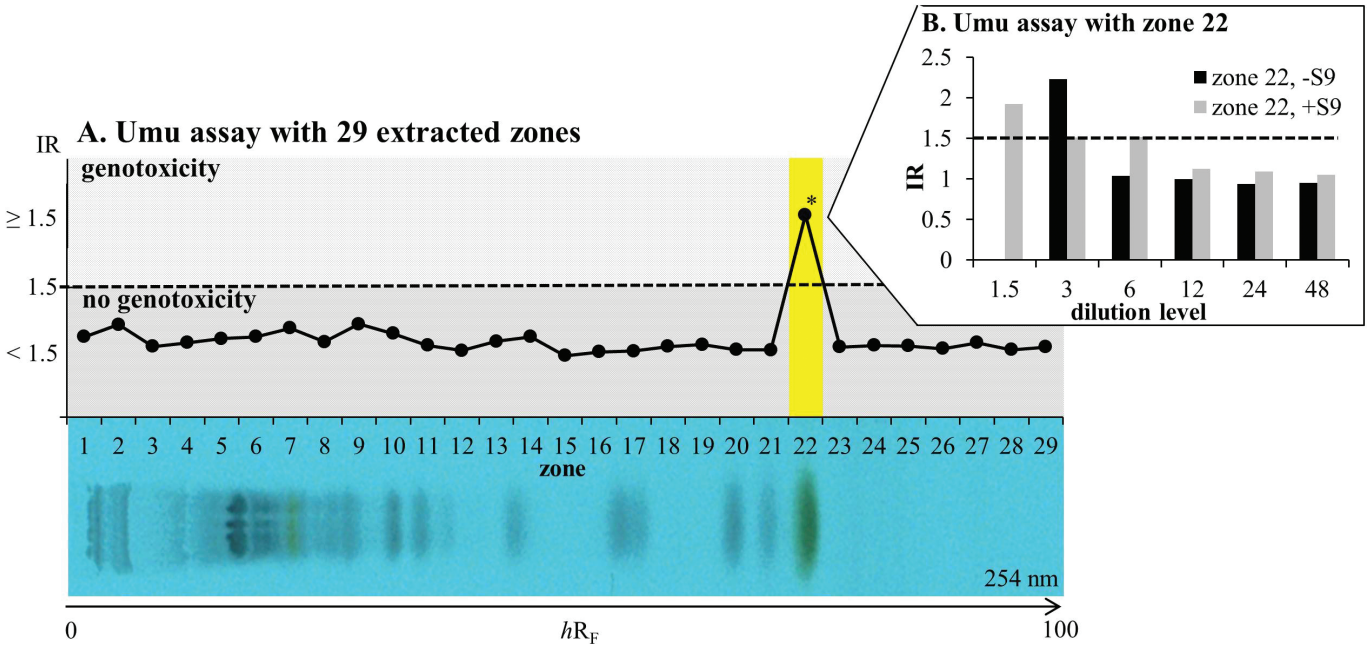
Figure 2

(A) Separation of MF and (B) separation and extraction of 29 zones from 10 to 80 mm of sample MF 4.

By the separation of a metformin standard (100 mg L^{-1}) with the gradient development, metformin achieved a hR_F of 5 **(Figure 2A).** After chlorination of metformin with hypochlorite, many bands over a broad separation distance could be detected. Two yellow zones occurred, which caused the yellow staining of the sample MF 4 (Figure 2B, arrows on zone 7 and zone 22). All 29 extracted zones were investigated with the umu assay (without dilution steps), but first, no genotoxic effect could be detected **(Figure 3A).** Only the yellow zone 22 (62.5 mm) caused a cytotoxic effect to *Salmonella typhimurium* TA1535/pSK1002. The GR of zone 22 without metabolic activation was only −0.1 and that with metabolic activation reached 0.2, while the GRs of all the other extracted zones were between 0.7 and 1.1. This finding indicates the intense inhibition of the bacteria in zone 22, as already mentioned from the investigation of the whole sample.

To check the effect of the yellow zone 22, the extraction of this zone was performed again. For the implementation of the umu assay in triplicate and with 6 dilution levels now, the extraction of zone 22 was repeated 12 times on one HPTLC plate to obtain a sufficient extract volume (2400 µL). Next, 2160 µL of extract was required for the umu assay with and without metabolic activation, and the remaining extract was used for LC–HRMS measurement. The extracted zone was already stained yellow; therefore, it could be ensured that the yellow substance in the zone could be (at least partly) extracted from the HPTLC plate with the used eluent (ultrapure water). This time, the umu assay showed a genotoxic effect for zone 22, both with and without metabolic activations. The maximal IR without metabolic activation reached 2.23 (LID = 6), and with metabolic activation, it was 1.92 (LID = 12). The dilution level of 1.5 without metabolic activation again was cytotoxic to the bacteria and therefore could not be evaluated (Figure 3B).

In comparison to the first investigation of zone 22, where in both approaches (−S9 and +S9 activation) no IR could be calculated because of cytotoxicity (not shown), with the second investigation, the GR of the umu assay at the dilution level of 1.5 with metabolic activation was sufficient for calculating the IR (Figure 3A* and B). However, the GR of 0.8 was still under the mean GR of 0.93 ± 0.08 (N = 10) for the remaining dilution levels, and thus the little cytotoxic effect was determined. Altogether, it became apparent that cytotoxicity occurs to a lesser extent with metabolic activation compared to trials without metabolic activation. It is likely that cytotoxic compounds were transformed through enzymes in the S9 mix. Since the S9 enzyme mix from rat liver is a very



*because of cytotoxicity in the first investigation (A), the IR (dilution level: 1.5, +S9) of the second investigation (B) is shown.

Figure 3

(A) IR of the umu assay with 29 extracted zones from the HPTLC plate at a dilution level of 1.5. (B) IR of the umu assay with zone 22 at different dilution levels. In the case of different IRs with and without metabolic activation, the larger IR is shown.

complex system, the transformational efficiency of cytotoxic compounds may fluctuate, which would explain the different GRs between different trials.

3.2.2 Combining EDA with HPTLC to Non-Target Screening

Since the genotoxic effect was matched to zone 22 through the extractions of 29 zones from the HPTLC plate, the identification of the effective compound was sought. Therefore, the sample MF 4 and the extracted zone 22 were measured *via* LC–HRMS. The blank, the control, and a blank extract from the HPTLC plate at the same hR_F as zone 2 (hR_F = 76) were used as blanks for the validation of the features in the non-target screening. The signals in the sample have to be 5 times higher than the mean of the blank signals to pass the feature validation. The assigned isotopes and adducts were not involved in further data evaluation.

After feature validation, 624 features remained for the sample MF 4. Zone 22 contained 194 valid features with an intersection with the sample MF 4 of 61 features **(Figure 4).**

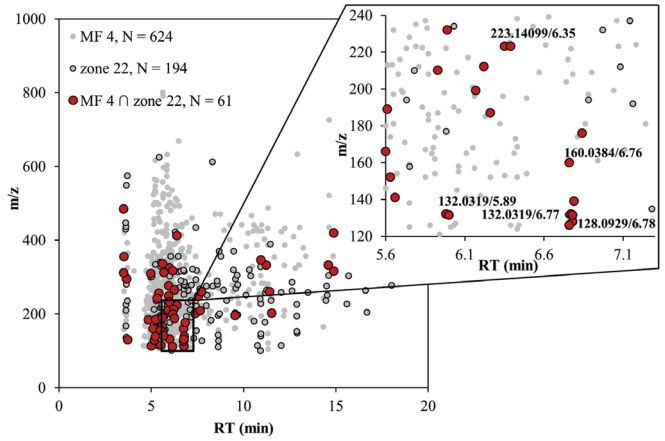


Figure 4

Scatter diagram with valid features of sample MF 4, zone 22, and the intersection of MF 4 with zone 22. The enlargement shows the 5 features with highest intensity in the intersection of sample MF 4 and zone 22.

Through the separation of the sample with HPTLC and the extraction from the plate, the number of features, which could be responsible for the genotoxic effect, was reduced by a factor of 10 from 624 to 61. Within the remaining 61 possible features, the effective substance was sought. Therefore, the features were sorted based on their intensity, and the most intense features were investigated first because they provide the most expressive isotopic patterns and MS/MS spectra. In **Table 3,** the five features with the highest intensities in zone 22 are shown.

The proposed formulas for the features with the highest intensity were matched based on the isotopic pattern and the respective MS/MS spectrum. The formulas with the highest accordance in both criteria were selected. In the literature, two of these formula proposals ($C_4H_6ClN_3$ and $C_4H_6ClN_5$) were already described as the TPs of the chlorine-treated metformin [26] **(Figure 5).**

Table 3

The five most intense features of the intersection of sample MF 4 and zone 22 (cps: counts per second).

m/z	RT [min]	Intensity MF 4 [cps]	Intensity zone 22 [cps]	Formula proposal
132.0319	5.98	524660	353463	$C_4H_6ClN_3$
132.0319	6.77	868898	90062	$C_4H_6ClN_3$
128.0929	6.78	253985	27476	$C_4H_9N_5$
160.0384	6.76	109494	10486	$C_4H_6ClN_5$
223.1409	6.35	599282	9107	$C_8H_{14}N_8$

Armbruster et al. [26] found that during the chlorination, metformin is degraded to a cyclic dehydro-1,2,4-triazole derivate with an intense yellow color. This TP accords to the found feature 160.0384/6.76, and the proposed formula of $C_4H_6ClN_5$. However, this reaction product is not that stable and is degraded to a colorless chloroorganic nitrile, which is suited to the features 132.0319/5.98 and 132.0319/6.77 with the proposed formula of $C_4H_6ClN_3$. Therefore, the occurrence of the two features with the same mass (132.0319) but with different retention times can be explained. One part of the yellow TP is already degraded to the colorless TP before LC separation and therefore occurs at another retention time (5.98 min). The other feature of the colorless TP with the same retention time as the yellow TP (6.77 min) is probably an in-source fragment, which is caused by the N_2-elimination in the ESI source.

For further confirmation, if one of these two products caused the genotoxic effect on the umu assay, recrystallized yellow

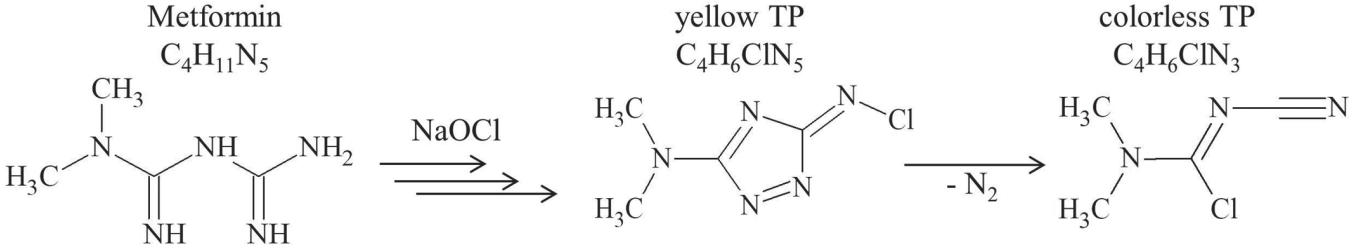


Figure 5

Degradation of metformin to a yellow and a colorless TP through sodium hypochlorite [26].

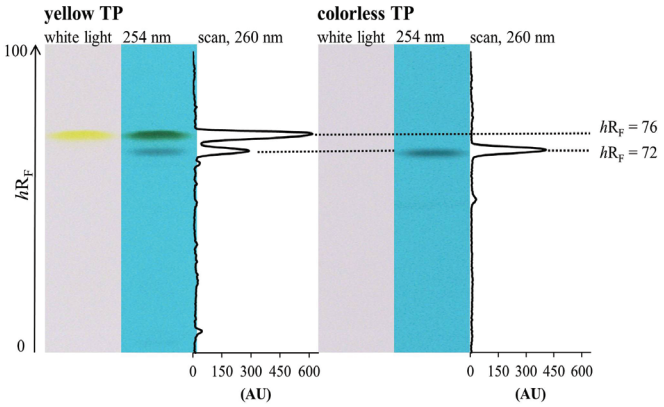


Figure 6

Purity review of the recrystallized yellow and colorless TP.

and colorless TPs were obtained from DVGW Technologiezentrum Wasser. The detailed procedure for the recrystallization is described in Ref. [26]. Both TPs were dissolved in ultrapure water (1 mg mL^{-1}), and their purities were reviewed with HPTLC separation (application volume 10 µL) **(Figure 6).**

The HPTLC gradient separation of the yellow TP occurred in two zones, whereby one zone showed a yellow staining and reached the same hR_F as zone 22 (hR_F = 76), and the second one was found just below the yellow zone. It reached the same hR_F as the colorless TP (hR_F = 72), which indicated that parts of the yellow TP were already degraded to the colorless TP.

Then, the umu assay was performed with both of the TPs (500 mg L^{-1}, without HPTLC separation), and it could be shown that the yellow one causes a genotoxic effect with and without metabolic activation, while the colorless TP only has a cytotoxic effect on the bacteria. The maximal IRs for the yellow TP were 5.82 (–S9, LID = 48, c_{LID} = 10 mg L^{-1}) and 3.37 (+S9, LID = 48, c_{LID} = 10 mg L^{-1}). Until reaching the dilution level of 6, the yellow TP is cytotoxic to the bacteria; therefore, the IR must not be calculated **(Figure 7).**

To review, if both the TPs can be eluted from the HPTLC plate with ultrapure water, the recoveries of the substances were determined. Therefore, both TPs (100 mg L^{-1}) were applied to the HPTLC plate in a 6 mm × 6 mm area with 50 µL each, and extraction with the TLC–MS Interface 2 was performed in the middle of this area. The required concentrations of 100% recovery were calculated over the elution head's area. These concentrations of the two TPs were doped to blank extracts from the HPTLC plate as references, and were set as 100% recovery. Based on their accurate masses in the LC–HRMS measurement, the recoveries of the TPs in the plate extracts could be determined. The recovery of the yellow TP was 106%, and for the colorless, TP it was 101%.

The final identification of the yellow TP in zone 22 was done *via* the comparison of the retention times, isotopic patterns, and the MS/MS spectra of the recrystallized yellow TP, the sample MF 4, and zone 22. The retention time of *m/z* 160.0384 was 6.8 min in all the 3 measured samples. The isotopic pattern of the *m/z* 160.0384 showed the typical chlorine isotopic pattern, and the MS/MS spectra were identical **(Figure 8).**

3.3 Quantification of the Yellow TP

The quantification of the yellow TP in the sample MF 4 was performed *via* HPTLC and the measurement of yellow absorption (400 nm) at hR_F = 76. Thereby, it must be considered that the yellow TP is not stable, such that only its concentration could be estimated. The polynomial calibration curve ($y = -1.188x^2 + 255.4x - 1025$, r = 0.99986, *%RSD* = 3.0%) was determined with 4 standard concentrations (0.039 mmol L^{-1}, 0.077 mmol L^{-1}, 0.39 mmol L^{-1}, and 0.77 mmol L^{-1}). The concentration of the yellow TP in the sample MF 4 was estimated as 0.43 mmol L^{-1} by applying 0.77 mmol L^{-1} MF at the beginning. Therefore, it could be said that through chlorination with 4 eq NaOCl, about a half of the applied MF is converted to the yellow TP.

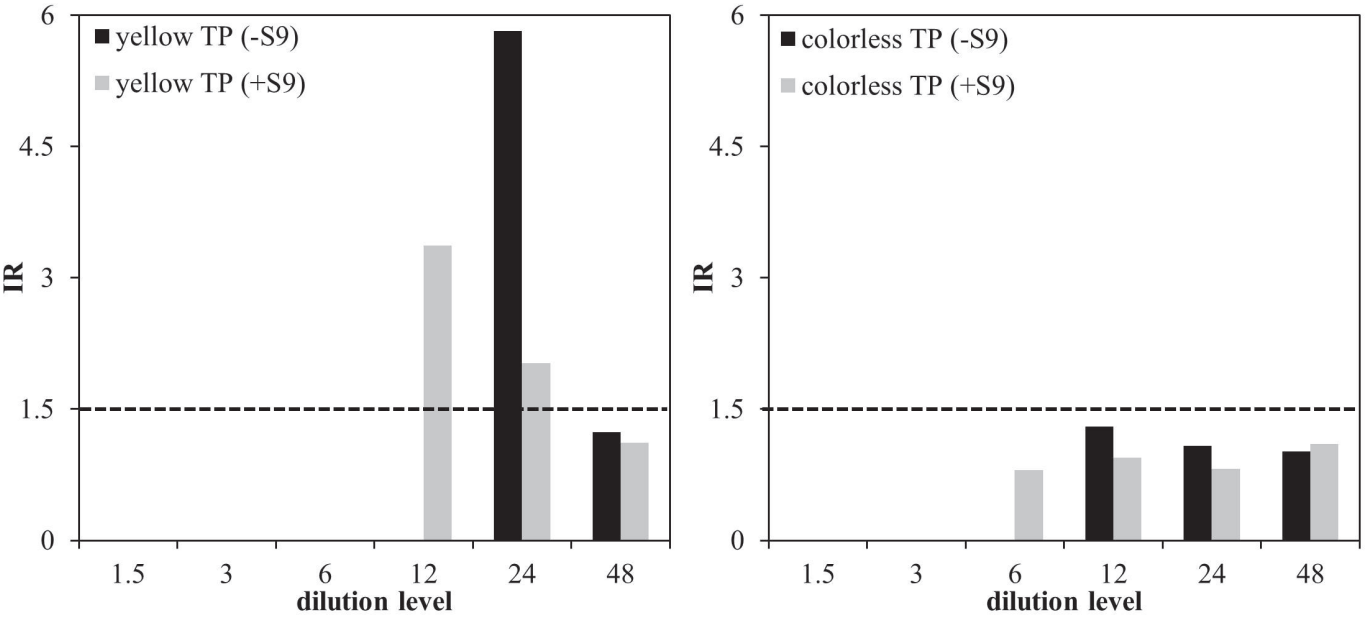


Figure 7

IR of the umu assay with the yellow and colorless TPs.

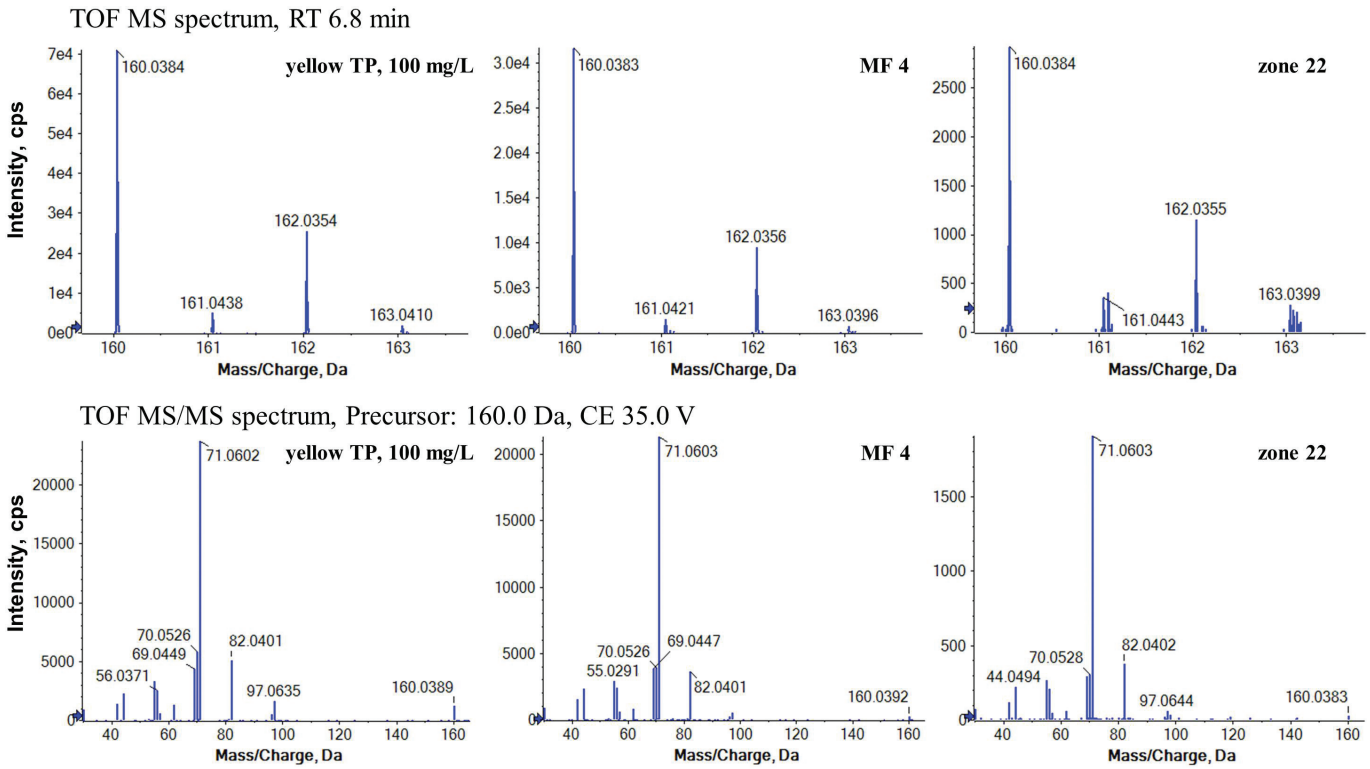


Figure 8

Comparison of MS and MS/MS spectra of the recrystallized yellow TP, sample MF 4, and zone 22.

4 Conclusion

In this study, EDA was performed by the coupling of HPTLC with biological detection in the microtiter plate using the umu assay. Since the umu assay currently cannot be directly applied on the HPTLC plate, the assay was performed according to DIN 38415-T3 on the microtiter plate. The difficulty of the coupling of HPTLC with a bioassay in the microtiter plate is the selection of the suitable zones for extraction with the TLC–MS Interface 2, because after HPTLC, the position of the effective zone on the plate is unknown. If no metainformation regarding the effective zone is available, the solution is the extraction of the whole retardation area.

This procedure was applied for a chlorinated metformin sample whereby a genotoxic effect was measured in the form the HPTLC plate extracted zones. By combining EDA with a non-target screening with LC–HRMS, finally a genotoxic compound could be identified. Thereby, the advantage of the separation before the biological detection became apparent, because the number of possible features for the identification of the compound could be narrowed down from 624 to only 61. The identification of the remaining 60 unknown features in the intersection of sample MF 4 and zone 22 would require further research, which is still a major challenge.

In further work, the developed workflow could be transferred to other bioassays in order to broaden the field of application. The advantage of the method is that standardized test systems in the microtiter plate can also be used directly without the need to transfer the bioassay to the HPTLC plate. Finally the developed method is intended to help the often complex identification of substances effective in bioassays and to simplify this process as much as possible.

Acknowledgments

The authors gratefully acknowledge Oliver Happel and Dominic Armbruster from DVGW Technologiezentrum Wasser (Karlsruhe, Germany) for the provision of the recrystallized yellow and colorless TP. Further acknowledgement goes to the Umweltbundesamt, Tamara Grummt, and Jochen Kuckelkorn, who enabled the implementation of the AMES fluctuation assay and the micronucleus assay with the chlorinated metformin sample.

References

[1] *M. Huerta-Fontela, M.T. Galceran, F. Ventura,* Water Res. **45** (2011) 1432–1442.

[2] *M.R. Boleda, M.T. Galceran, F. Ventura,* Environ. Pollut. **159** (2011) 1584–1591.

[3] *U. von Gunten,* Water Res. **37** (2003) 1469–1487.

[4] *M.d.M. Gómez-Ramos, M. Mezcua, A. Agüera, A.R. Fernández-Alba, S. Gonzalo, A. Rodríguez, R. Rosal,* J. Hazard. Mater. **192** (2011) 18–25.

[5] *M.C. Dodd, H.-P.E. Kohler, U. von Gunten,* Environ. Sci. Technol. **43** (2009) 2498–2504.

[6] *K. Li, A. Yediler, M. Yang, S. Schulte-Hostede, M.H. Wong,* Chemosphere **72** (2008) 473–478.

[7] *W. Brack, S. Ait-Aissa, R.M. Burgess, W. Busch, N. Creusot, C. Di Paolo, B.I. Escher, L. Mark Hewitt, K. Hilscherova, J. Hollender, H. Hollert, W. Jonker, J. Kool, M. Lamoree, M. Muschket, S. Neumann, P. Rostkowski, C. Ruttkies, J. Schollee, E.L. Schymanski, T. Schulze, T.-B. Seiler, A.J. Tindall, G. De Aragão Umbuzeiro, B. Vrana, M. Krauss,* Sci. Total Environ. **544** (2016) 1073–1118.

[8] *W. Brack,* Anal. Bioanal. Chem. **377** (2003) 397–407.

[9] *T. Grummt, J. Kuckelkorn, A. Bahlmann, C. Baumstark-Khan, W. Brack, T. Braunbeck, S. Feles, S. Gartiser, H. Glatt, R. Heinze, C. Hellweg, H. Hollert, R. Junek, M. Knauer, B. Kneib-Kissinger, M. Kramer, M. Krauss, E. Kuster, S. Maletz, W. Meinl, A. Noman, E.-M. Prantl, E. Rabbow, R. Redelstein, P. Rettberg, W. Schadenboeck, C. Schmidt, T. Schulze, T.-B. Seiler, L. Spitta, D. Stengel, P. Waldmann, A. Eckhardt,* Environ. Sci. Eur. **25** (2013) 27.

[10] German standard methods for the examination of water, waste water and sludge – Sub-animal testing (group T) – Part 4: Determination of the genotoxic potential using the Salmonella microsome test (Ames Test) (T 4) (DIN 38415-4:1999-12), Beuth Verlag, Berlin, 1999.

[11] Water quality – Evaluation of genotoxicity by measurement of the induction of micronuclei – Part 2: Mixed population method using the cell line V79 (ISO 21427-2:2006); German version EN ISO 21427-2:2009, Beuth Verlag, Berlin, 2009.

[12] German standard methods for the examination of water, waste water and sludge – Sub-animal testing (group T) – Part 3: Determination of the genotype potential of water and waste water components with the umu-test (T 3) (DIN 38415-3:1996-12), Beuth Verlag, Berlin, 1996.

[13] *Y. Oda, S.-i. Nakamura, I. Oki, T. Kato, H. Shinagawa,* Mutat. Res. Environ. Mutagen. Relat. Subj. **147** (1985) 219–229.

[14] *X. Ouyang, P.E.G. Leonards, Z. Tousova, J. Slobodnik, J. de Boer, M.H. Lamoree,* Anal. Chem. **88** (2016) 2353–2360.

[15] *F. Itzel, K.S. Jewell, J. Leonhardt, L. Gehrmann, U. Nielsen, T.A. Ternes, T.C. Schmidt, J. Tuerk,* Sci. Total Environ. **624** (2018) 1443–1454.

[16] *G. Morlock, W. Schwack,* J. Chromatogr. A **1217** (2010) 6600–6609.

[17] *S.C. Weiss, N. Egetenmeyer, W. Schulz,* in: *G. Reifferscheid, S. Buchinger* (eds), *In vitro* Environmental Toxicology – Concepts, Application and Assessment, Springer, Berlin, Heidelberg, 2016, 187–224.

[18] *I. Klingelhöfer, G.E. Morlock,* J. Planar Chromatogr. **30** (2017) 411–417.

[19] *K. Burger,* Chem. Plant Prot. **12** (1995) 181–195.

[20] *C. Stiefel, S. Dietzel, M. Endress, G.E. Morlock,* J. Chromatogr. A **1462** (2016) 134–145.

[21] *L. Stütz, S.C. Weiss, W. Schulz, W. Schwack, R. Winzenbacher,* J. Chromatogr. A **1524** (2017) 273–282.

[22] *Á.M. Móricz, P.G. Ott, T.T. Häbe, A. Darcsi, A. Böszörményi, Á. Alberti, D. Krüzselyi, P. Csontos, S. Béni, G.E. Morlock,* Anal. Chem. **88** (2016) 8202–8209.

[23] *N. Egetenmeyer, S.C. Weiss,* J. Liq. Chromatogr. Relat. Technol. **40** (2017) 69–74.

[24] *G. Morlock, W. Schwack,* TrAC–Trend. Anal. Chem. **29** (2010) 1157–1171.

[25] *M. Scheurer, F. Sacher, H.-J. Brauch,* J. Environ. Monit. **11** (2009) 1608–1613.

[26] *D. Armbruster, O. Happel, M. Scheurer, K. Harms, T.C. Schmidt, H.-J. Brauch,* Water Res. **79** 104–118.

[27] Chemicals used for treatment of water intended for human consumption – Sodium hypochlorite; German version EN 901:2013 (DIN EN 901:2013), Beuth Verlag, Berlin, 2013.

[28] *T. Bader, W. Schulz, K. Kümmerer, R. Winzenbacher,* Anal. Chim. Acta **935** (2016) 173–186.

Ms received: November 20, 2018
Accepted: January 29, 2019

Publication 3

L. Stütz, W. Schulz, R. Winzenbacher, Identification of acetylcholinesterase inhibitors in water by combining two-dimensional HPTLC and high-resolution mass spectrometry, *Journal of Chromatography A*, 1624 (2020) 461239, https://doi.org/10.1016/j.chroma.2020.461239.

Journal of Chromatography A 1624 (2020) 461239

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Identification of acetylcholinesterase inhibitors in water by combining two-dimensional thin-layer chromatography and high-resolution mass spectrometry

Lena Stütz[a,b,*], Wolfgang Schulz[a], Rudi Winzenbacher[a]

[a] Laboratory for Operation Control and Research, Zweckverband Landeswasserversorgung, Am Spitzigen Berg 1, 89129 Langenau, Germany
[b] Institute of Food Chemistry, University of Hohenheim, Garbenstraße 28, 70599 Stuttgart, Germany

ARTICLE INFO

Article history:
Received 29 March 2020
Revised 11 May 2020
Accepted 12 May 2020
Available online 27 May 2020

Keywords:
Effect-directed analysis
TLC-MS Interface
Non-target screening
Intersection formation
Prioritization

ABSTRACT

Effect-directed analysis (EDA) is increasingly used in environmental monitoring to detect and identify key toxicants. High-performance thin-layer chromatography (HPTLC) has proven to be a very suitable fractionation technique for this purpose. However, HPTLC is limited in its separation efficiency. Thus, separated fractions could still contain many different components and identification of the effective substances remains difficult. Therefore, in this study a workflow for selective EDA with two-dimensional HPTLC in combination with high-performance liquid chromatography-high-resolution mass spectrometry (HPLC-HRMS) was developed. The aim of the workflow was the stepwise reduction of the sample complexity in order to reduce the number of signals that could be responsible for the measured effects. As a consequence, the identification of effective substances should be facilitated. The acetylcholinesterase inhibition assay (AChE assay) for the detection of potential neurotoxic compounds was applied for biotesting. The transfer of effective zones from the first to the second dimension and also to the mass spectrometric measurement was enabled by extraction.

A proof of concept was performed by spiking six acetylcholinesterase inhibiting substances into three different water matrices that were investigated with the developed workflow. The successful prioritization of all spiked compounds confirmed the efficiency of the workflow, regardless of the sample matrix. Biotesting of different water samples resulted in numerous potentially neurotoxic effects, which overlapped strongly in the first separation dimension. The higher peak capacity reached by two-dimensional HPTLC, on the other hand, resulted in discrete effective zones and enabled the identification of several compounds. For the substances lumichrome, a derivate of riboflavin and paraxanthine as well as for linear alkylbenzene sulfonates that were applied as anionic surfactants in detergents, the inhibiting effect to the AChE could be confirmed.

1. Introduction

Protecting the aquatic environment and providing people around the world with safe drinking water are main challenges of our time [1]. Therefore, monitoring of the aquatic environment, also serving as a source of raw water for drinking water production, is of particular importance. Since aquatic environmental samples are complex substance mixtures their characterization and assessment remains challenging [2,3]. Often, target analysis is used to detect and to quantify known substances in water samples sensitively and accurately [4,5]. However, target analysis is not sufficient to capture the multitude of substances in environmental mixtures, especially because it is not able to capture the unknown compounds [6]. But there are substances, which have not been characterized yet and many transformation products currently are not known. To be able to detect also the unknowns, non-target screening has to be applied [7,8], such that a more comprehensive description of environmental samples becomes possible. In addition to the identity of substances and their transformation products, to date only little is known about their effects on humans and the ecosystem they come in contact with [9]. Therefore, effect-directed analysis (EDA) can be applied, which combines biotesting

* Corresponding author at: Laboratory for Operation Control and Research, Zweckverband Landeswasserversorgung, Am Spitzigen Berg 1, 89129 Langenau, Germany.
E-mail addresses: Stuetz.L@lw-online.de (L. Stütz), Schulz.W@lw-online.de (W. Schulz), Winzenbacher.R@lw-online.de (R. Winzenbacher).

https://doi.org/10.1016/j.chroma.2020.461239
0021-9673/

with a fractionation and chemical analysis [10]. The aim of the EDA is the detection of effects and the identification of effective compounds.

If an environmental sample causes an effect in a bioassay, the challenge is to evaluate the relevance of the contained compounds and select the important ones, namely the key toxicants [11,12]. With EDA, the goal of prioritizing and identifying relevant key toxicants by using fractionation steps can be pursued, so that environmental samples are divided into less complex fractions [13,14]. Only the effective fractions have to be further investigated to identify the respective bioactive compounds [15].

High-performance thin-layer chromatography (HPTLC) has proven to be a particularly suitable fractionation technique for EDA [16,17]. In contrast to column chromatography, HPTLC is an open separation system and since the layer is solvent-free after the separation, the *in-vitro* bioassay can be applied directly on the HPTLC plate [18]. But compared to high-performance liquid chromatography (HPLC), the achievable peak capacity of HPTLC is limited [10]. In a previous work, a selective two-dimensional (2D) HPTLC approach with use of the acetylcholinesterase inhibition assay (AChE assay) for the detection of potential neurotoxic effects in water samples was thus developed [19]. The aim of the work was to increase the peak capacity via a 2D HPTLC separation that results in a reduction of the number of substances in an effective zone. The characteristic of the developed approach was the transfer of only effective zones from the first to the second dimension by extraction (heart-cut approach) and was therefore called selective EDA with 2D HPTLC. Fractions without effects could already be excluded after the first dimension (1D). The AChE assay was applied twice, after the first and after the second separation dimension. Five mobile phases (MP) were developed for separation in the 2D, which were adapted to the retardation factor of the effective zone in the 1D. Thus, the eluent strength of the MP in the 2D was optimally adjusted and a peak capacity of 208 compared to the previous 29 in one-dimensional gradient development was achieved [19].

When environmental samples are investigated with EDA and unknown effective substances should be identified, additional structure elucidation has to be performed. Therefore, in the current study the selective EDA using the described 2D HPTLC approach was connected to high-performance liquid chromatography (HPLC) with high-resolution mass spectrometry (HRMS) and a non-target screening (NTS). Approaches that combine 2D separations with bioassays and mass spectrometry are already known from the literature [14,20]. However, either 2D HPLC separations or a combination of 2D HPTLC + HPLC separation were used, whereby in both approaches the bioassay is applied only once. In one study, EDA was performed with a 2D HPTLC separation [21]. However, no additional analysis for structure elucidation was performed, so that only known effective substances could be determined on the basis of their retardation factors.

In this study, a workflow for selective EDA with 2D HPTLC in combination with HPLC-HRMS was developed with the aim to prioritize and identify effective compounds. With HPTLC separation and the AChE assay in two dimensions, many components of the sample were separated from the effective zone and therefore could already be excluded as cause for the effect. The samples were investigated via NTS and a prioritization was performed by forming the intersection of the signals detected in the sample and the corresponding plate extracts. Thus, a detailed and elaborate examination of the individual remaining signals, with the aim of identifying the effective substance(s), was enabled. In the first part of the work, the developed workflow was evaluated and a proof of concept with spiked water samples was carried out. Subsequently, different water samples were examined via EDA with 2D HPTLC in combination with HPLC-HRMS regarding their potential neurotoxic effects and several substances could be identified in the effective zones.

2. Materials and methods

2.1. Chemicals and reagents

Ultrapure water was produced with an ultrapure water system (Purelab® Ultra, Elga LabWater, Lane End, UK). Acetonitrile (Rotisolv® ≥ 99.95%, LC-MS-Grade), acetone (Rotisolv® ≥ 99.9%, HPLC), water for LC-MS analysis (Rotisolv® Ultra LC-MS) and 2-propanol (Rotisolv® ≥ 99.95%, LC-MS-Grade) were purchased from Carl Roth (Karlsruhe, Germany). Dichloromethane (Ultra Resi-analyzed®, ≥ 99.8%) and n-hexane (Ultra Resi-analyzed, ≥ 99.5%) were purchased from J.T. Baker (Center Valley, US). Methanol (AMD Chromasolv™ ≥ 99.9% and Rotisolv® ≥ 99.95%, LC-MS-Grade) was supplied by Sigma-Aldrich (Steinheim am Albuch, Germany) and Carl Roth. Chloroform (HiPerSolv Chromanorm®) and ammonia (25%, AnalaR Normapur®) were purchased from VWR International (Bruchsal, Germany), while formic acid (98 - 100%), ethyl acetate (Uvasol®) and toluene (Uvasol®) were supplied by Merck (Darmstadt, Germany).

For the AChE assay, acetylcholinesterase Type VI-S (2000 U/vial) from electric eel, ascorbic acid and 3-indoxyl-3-acetate from Sigma-Aldrich were used. Bovine serum albumin, hydrochloric acid (32%) and 2-amino-2-(hydroxymethyl)-1,3-propanediol were supplied by Merck.

2.2. Standards

The stability of HPTLC was examined with a chromatography control mix consisting of nine substances (1,3,6-naphthalenesulfonic acid, 1,5-naphthalenesulfonic acid, 1-naphthalenesulfonic acid, theobromine, caffeine, thiourea, N-phenylacetamide, benzamide, and N,N-dimethyl-4-[(E)-phenyldiazenyl]-aniline) with 10 mg/L each.

89 substances were investigated regarding their recovery after solid phase extraction (SPE) and after extraction with the TLC-MS Interface (Table S 1). The proof of concept of the developed workflow was performed by spiking six substances with potential neurotoxic effects to three water samples (Table 1). For final confirmation of identified substances, reference standards were purchased: clopidogrel carboxylic acid (TRC, North York, Canada, 97.0%), metolachlor ethanesulfonic acid (Neochema, Bodenheim, Germany), sodiumdodecylbenzenesulfonate (Sigma-Aldrich, 49.7%, mixture of homologues C10-C13 and isomers), triamterene (TRC, 98.0%), lumichrome (Cayman Chemical, Ann Arbor, US, 98.1%), methocarbamol (Sigma-Aldrich, 99.8%), oxazepam (Neochema), phenazone (Dr. Ehrenstorfer, Augsburg, Germany, 99.0%) and paraxanthine (Cayman Chemical, 98.0%).

2.3. Sampling and sample preparation

Grab samples of drinking water and surface waters were taken with 1 L each. Sewage waters were sampled as grab samples after the first treatment stage consisting of a grit chamber and a grease trap. In the following, the sample is referred to as untreated sewage water. The second sampling was done after the final treatment of the purified sewage water with powdered activated carbon (purified sewage water).

Enrichment was performed by solid phase extraction (SPE) [22,23]. The enrichment factor for drinking, surface and purified sewage water was 1000. In order to prevent clogging of the SPE cartridge, the untreated sewage water was enriched by a factor of 100 due to the higher matrix load of the sample. Two pH-values

Table 1
Overview of the spiked substances.

Substance	$\overline{hR_F}$ (1D)	hR_F area (2D)	$\overline{hR_F}$ (2D)	Formula	log K_{OW} (EPIWEB 4.1)	Class	ß, spiked (µg/L)
Azamethiphos	61	D	17	$C_9H_{10}ClN_2O_5PS$	1.00	Pesticide (Thiophosphoric acid ester)	500
Caffeine	43	C	8	$C_8H_{10}N_4O_2$	0.16	Drug (Alkaloide)	41.5
Donepezil	25	B	56	$C_{24}H_{29}NO_3$	4.86	Pharmaceutical (Piperidinderivate)	40.7
Galanthamine	14	A	27	$C_{17}H_{21}NO_3$	2.29	Pharmaceutical (Plant alkaloid)	46.9
Methiocarb-sulfoxide	39	B	64	$C_{11}H_{15}NO_3S$	0.70	Metabolite pesticide (Carbamate)	3.0
Paraoxon-ethyl	69	D	47	$C_{10}H_{14}NO_6P$	1.97	Metabolite pesticide (Phosphoric acid ester)	1.7

(pH 2 and pH 7) were applied for investigation of the SPE recovery. Therefore the samples were adjusted to these pH values with HCl or NaOH, respectively. Due to higher substance recoveries at pH 7, only this pH value was used for the enrichment of the water samples.

Bond Elut Plexa cartridges (500 mg, Agilent Technologies, Santa Clara, US) containing a non-polar divinylbenzene-based neutral polymeric sorbent were used for the enrichment. The cartridges were conditioned with each 5 mL n-hexane, dichloromethane, acetone, methanol and ultrapure water. The sample was filled in a storage vessel and ran over the cartridge, powered by a peristaltic pump with a flow of 2.5 mL/min. After loading, the cartridges were dried for 45 min in an airstream. Three solvents were used for elution. At first, 3 mL methanol (0.4% v/v ammonia) was used. The second eluent consisted of 4 mL of a 50:50 v/v mixture of methanol (0.4% v/v ammonia) - ethyl acetate, and the third eluent was dichloromethane (3 mL). Evaporation was performed in a gentle stream of nitrogen after respectively 1 mL of solvent was eluted. 50 µL of ultrapure water was added as a keeper before evaporation of dichloromethane. The residue (about 50 µL of ultrapure water) was diluted with 1 mL methanol. Additionally, a blank of the SPE cartridge was prepared. Therefore, the cartridge was conditioned and eluted the same way as the sample cartridge but without any contact with a sample.

2.4. High-performance thin-layer chromatography

2.4.1. HPTLC apparatus and software

For application, separation and detection, the Automatic TLC Sampler 4 (ATS 4), Automatic Developing Chamber 2 (ADC 2), Automated Multiple Development 2 (AMD 2), TLC Scanner 4 and TLC Visualizer were used. The associated software was winCATS (1.4.9). Elution of zones from the HPTLC plate was performed with the TLC-MS Interface 2 with an oval elution head (4 mm × 2 mm). Immersion of the plates in the AChE solution was done with the Chromatogram Immersion Device 3 (all from CAMAG, Muttenz, Switzerland). For the AChE assay additionally the spraying apparatus, Derivatizer from CAMAG and an incubator at 37 °C (VWR International) were used.

2.4.2. HPTLC chromatography

Before use, HPTLC LiChrospher silica gel 60 F_{254S} plates (Merck) were immersed twice in 2-propanol for 20 min. After drying at 120 °C on the plate heater, the plates were predeveloped to the top edge with acetonitrile. Finally, the plates were heated again to 120 °C for 20 min. Cleaned plates were stored in a desiccator at room temperature. This procedure was necessary to remove plate contaminations interfering with the bioassay detection.

The sample application to the HPTLC plate was performed with the ATS 4. 30 µL of the control mix for chromatography was applied. 50 µL of each sample and SPE blank extract was applied in the 1D as area application to reduce matrix effects (6 mm × 3 mm). In order to minimize the substance loss, the extracted zones were applied with the entire extracted volume (200 µL, sufficient extract volume by extracting the zones three times from the plate) for 2D separation as band application (6 mm × 1 mm).

For separation in the 1D the automated multiple development system (AMD 2) was used. The plate was developed in a stepwise manner with increasing migration distances and decreasing eluent strength. The first three separation steps were necessary to focus the substances on the application zone to thin bands. As solvents, methanol (0.05% v/v formic acid), dichloromethane and n-hexane were used (Table S 2). A dynamic approach was used for separation in the 2D, where only the selected effective zones of the 1D were applied and separated again on the same stationary phase material but with a different mobile phase (MP). The detailed procedure is described in [19]. For this approach five arbitrary retardation areas were appointed in equal intervals and for each of these areas a different MP was applied (Table S 3). The MP used in the 2D was selected dependent on the apex hR_F of the effective zone in the 1D.

After the separation, images were taken from the HPTLC plate with the TLC Visualizer (254 nm, 366 nm, white light) and a multi wavelength scan at seven wavelengths (190 nm up to 300 nm) was recorded. The quality control of the HPTLC separations in both dimensions was performed using the hR_F of the substances in the chromatography control mix (Table S 4). Before the application of the bioassay, the HPTLC plates were adjusted to a neutral pH. Therefore, the plates were placed for 10 s in NH_3-vapor and then put under vacuum (10 min) to remove the remaining NH_3 (pH after neutralization approx. 7.5).

2.5. Acetylcholinesterase inhibition assay with HPTLC (AChE assay)

The AChE assay was performed as described in [19] with few modifications. 20 g/L of 3-idoxyl-3-acetate dissolved in methanol was used as a substrate to evaluate the activity of the enzyme. When the enzyme was active, the substrate was cleaved to indoxyl and reacted with oxygen to indigo white. Both reaction products could be measured at 366 nm because of their blue fluorescence.

The AChE assay starts with the immersion of the plate in the enzyme solution (Chromatogram Immersion Device 3, dipping time 1 s, dipping speed 5 cm/s). The supernatant enzyme solution was carefully pulled off with a squeegee. The plate was incubated in an incubator for 5 min at 37 °C with humidity higher than 90%. After 6.5 min, the substrate solution was sprayed to the plate with the spraying apparatus (amount of reagent: 600 µL, nozzle: green, level: 5). 8.5 min after the immersion, the fluorescence detection of the enzyme inhibition started (wavelength: 366 nm, exposure time: 200 ms). A sequence capturing 10 pictures in 10 min was gathered, where always the third picture was used for evaluation to obtain comparable results.

The evaluation of the results of the AChE assay was performed by means of an image evaluation with in-house MATLAB scripts (R2017a, The MathWorks, Inc., Natick, US). For this purpose, the blue channel of the color image was read out (B) and the blue levels of the sample tracks, B(sample) and the corresponding background tracks, B(BG), were determined. The effect was calculated

for each point of the hR_F range using the following formula:

$$effect_{hR_F} = \left(B(BG)_{hR_F} - B(sample)_{hR_F}\right) / B(BG)_{hR_F}$$

After the evaluation, a chromatogram could be generated in which the effect was plotted against the hR_F.

2.6. Extraction of effective zones

During the workflow, the TLC-MS Interface was required twice for extraction of effective zones. In the first step, the zones have to be transferred from the 1D to the 2D for further separation and biotesting. After the 2D the zones were extracted again and a non-target screening was performed.

A challenge is to determine the coordinates of the effective zone on the HPTLC plate correctly. One reason for this is that the extraction cannot be performed after the bioassay has been applied, because the biological system would interfere with the second HPTLC separation and with the HPLC-HRMS detection. But separations on different HPTLC plates for the biotesting and the extraction cause variability of the retardation factors ($hR_F \pm 1.7$) and it becomes more difficult to hit the effective zones correctly. Fluctuations of the retardation factors within an HPTLC plate, however, are significantly lower. Therefore, all samples were applied four times to one HPTLC plate. After the separation in the 1D, the plate was cut in two parts, one part for the biotesting (one sample track) and the other part for the extraction of the effective zones (three sample tracks). Thus, the coordinates of the effective zone could be determined within one separation and variations between the separations were reduced. Each effective zone in the 1D was extracted three times, twice for the application in the 2D (again biotesting and extraction) and once for the HPLC-HRMS measurement. In order to ensure a uniform composition, the three extracts were mixed before their further use.

Since methanol has the highest eluent strength following water, it was used as the extraction eluent. Water, on the other hand, dissolves the silica layer and therefore more often leads to a leakage of the elution head. The substances in the target zone were eluted for 1 min with a flow of 200 µL/min [24]. To avoid a carry-over between extractions, the elution head was purged after each extraction with methanol for at least three minutes. Additional to the effective zone, always a blank from the HPTLC plate was required. The blank extraction was performed on an empty track on each HPTLC plate with the $hR_F = 50$.

2.6.1. Investigations of the recovery with the TLC-MS Interface

Recovery of the extraction with the TLC-MS Interface was investigated in two approaches. The first approach was to check the extent to which the substances can be dissolved from the HPTLC plate without considering how accurate the zones were hit with the extractor. Therefore a mix standard containing 89 substances was applied to the HPTLC plate in a 6 mm × 6 mm area and the extraction with the TLC-MS Interface was performed in the middle of this area. 10 ng of each substance in the mix was applied to the area of 36 mm^2, such that with the elution head (halved circle in the middle of which a rectangle is inserted, 7.14 mm^2) a maximal substance quantity of 1.98 ng could be extracted. This amount was set as 100% recovery and was spiked to a blank extract from the HPTLC plate. Based on their signal intensities in the HPLC-HRMS measurement, the recoveries of the 89 substances were determined. This examination was repeated four more times and the mean value of the data is shown in Table S 1.

In the second approach, the geometry of the elution head and the band geometry were considered. Therefore, the mix standard was applied in a 6 mm x 1.9 mm area, since 1.9 mm is the average peak width after the used gradient development in the AMD 2 [19] and 6 mm is used as the standard band length in our laboratory. The extraction was performed in the same way, as in the first approach and was repeated four more times. The mean value of the data is shown in Table S 1. Due to the geometry of the elution head, a maximum recovery of 62% can be expected under these conditions (peak width 1.9 mm and band length 6 mm).

It should be taken into account that the extraction of non-separated zones from the HPTLC results in an evenly distributed substance concentration within the zones. However, if a chromatographic separation is carried out beforehand, a Gaussian distribution of the substance concentrations is to be expected. Furthermore, the depth profile of the substances resulting from the HPTLC separation could also have an influence on the recovery of the extraction and is not considered in this investigation.

2.7. HPLC-QTOF-MS

Prior to injection (injection volume 95 µL), the sample extracts and the extracts from the HPTLC plate were diluted by a factor of 1:9 in ultrapure water each. HPLC-HRMS analysis was performed with the Shimadzu Nexera Prominence LC (Kyoto, Japan) coupled to a Quadrupole-Time-of-flight-mass spectrometer (QTOF-MS X500R, Sciex, Framingham, US). Each sample was analyzed in triplicate using reversed phase separation (Zorbax Eclipse Plus C18, 2.1 mm x 150 mm, 3.5 µm, Agilent, Santa Clara, US) with a flow of 0.3 mL/min. An HPLC gradient, consisting of A: water (0.1% v/v formic acid) and B: acetonitrile (0.1% v/v formic acid) with the following parameters was used: 2% B (0 min), 2% B (1 min), 20% B (2 min), 100% B (16.5 min), 100% B (22 min), 2% B (22.1 min), 2% B (33 min). For ionization, electrospray ionization (ESI) was used in the positive and negative mode and ions were monitored within the mass range of m/z 100 up to 1200. The acquisition of the MS^2 spectra was performed using data dependent acquisition (DDA) experiments for a maximum of 12 precursors with the highest intensity (after dynamic background subtraction) within one cycle. After fragmentation by collision-induced dissociation (CID) with the collision energy ramped from 20 V to 55 V, fragment ions were acquired between m/z 30–1200. The MS1 and MS2 experiments result in a total cycle time of roughly 1.1 s. Considering an average chromatographic peak width of about 10 s, the number of data points across the peak is still satisfying.

The data acquisition was done via the Sciex OS (1.2) software. For non-target screening, the MarkerView (1.2.1) software was used for finding and aligning the peaks across different samples and replicates and the Sciex OS (1.2) software (analytics tool) was required for the peak integration. All further processing steps were performed with in-house MATLAB scripts (R2017a, The MathWorks, Inc., Natick, US). The detailed procedure of the used non-target evaluation is described in [25].

After evaluation of non-target signals, validated features were prioritized by mathematical intersection formation. It was assumed that a substance causing an effect in the 1D and 2D occurs in the extracted zones of both dimensions and also in the sample extract. Thus, the intersection of the signals in the sample extract, the 1D zone and the 2D zone was formed and only signals detected in all three samples were considered for further identification attempts.

The first step in the identification of effective substances is the allocation of the exact masses to possible molecular formulas based on the isotope pattern and fragment information. By database searches, possible structural formulas were selected and reviewed based on their isotope patterns and MS/MS spectra. For clear identification of the substances, a comparison with the reference standard was performed. Concentrations of identified substances were determined by HPLC-QTOF-MS via an external eight-point calibration (0.01 µg/L up to 100 µg/L). In case of higher sub-

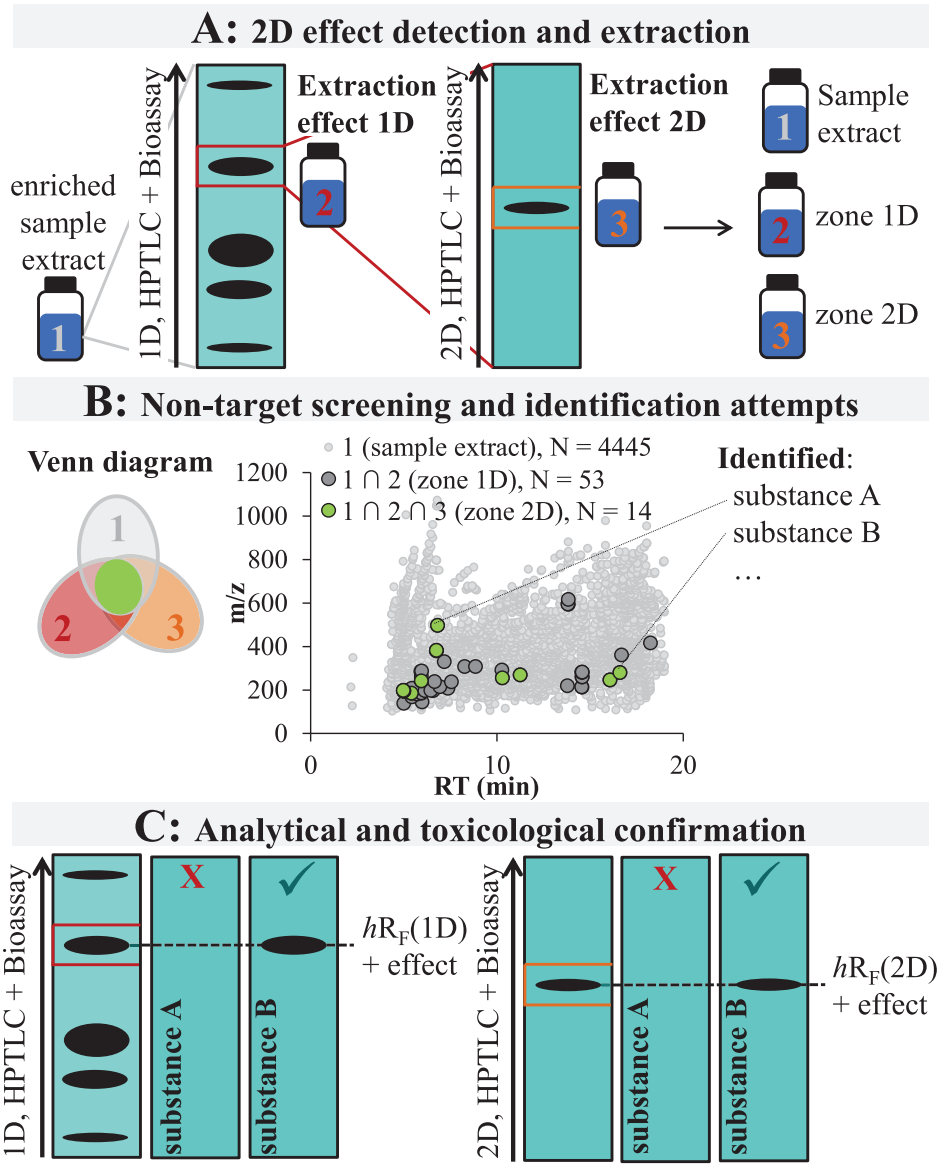


Fig. 1. Workflow of selective EDA with 2D HPTLC in combination with HPLC-HRMS.

stance concentrations, the sample extracts were diluted accordingly.

3. Results and discussion

3.1. Workflow of selective two-dimensional EDA with HPTLC and coupling to HPLC-HRMS

In this work, the identification of effective compounds detected via EDA should be facilitated through 2D separation and stepwise reduction of possible candidate signals. Therefore, a workflow combining selective EDA with 2D HPTLC in combination to HPLC-HRMS was developed. Effective zones of the 1D were extracted and applied to a HPTLC plate for second separation and biotesting. Effective zones in the second biotesting were extracted again (Fig. 1, Part A). This procedure provided three samples of each detected effect for the HPLC-HRMS measurement: the sample extract (1), the extracted zone of the 1D (2) and the extracted zone of the 2D (3).

Non-target screening and evaluation was performed as described in [25,26]. Mathematical intersections were formed to prioritize the signals that occurred in the sample extract, in the extracted zone of the 1D and also in the extracted zone of the 2D but not in the SPE and the HPTLC plate blank (Fig. 1, Part B). In a first step, the mathematical intersection of the sample extract and the extracted zone after 1D separation was formed (sample extract ∩ zone 1D). In the next step, the second separation dimension was included by forming the intersection of sample extract, extracted zone of the 1D and extracted zone of the 2D (sample extract ∩ zone 1D ∩ zone 2D). The procedure for identification of effective substances was adopted from previously published studies [8,12,27]. In short, it was tried to allocate the features in the intersection to the molecular formulas and then possible candidate structures were obtained by database searches. For identification, a comparison with a reference standard was performed.

If a substance could be identified via a reference standard, the hR_F and also the effect of the substance in the 1D and 2D were checked. Quantification of the identified substances was performed in the sample extract. Finally, the explainable portion of the effect in the sample extract was determined on the basis of the substance concentrations (Fig. 1, Part C).

3.1.1. Recovery of 89 substances in the developed workflow

A total of three extractions are carried out in the developed workflow. It has to be ensured that the extractions can be performed repeatedly and deliver good recoveries for substances in a broad polarity range. Therefore, 89 water relevant substances were selected and examined for their recoveries during the extractions. The substances covered a broad log K_{ow} range (EPIWEB, 4.1, US EPA) from -2.34 (metoprolol acid) up to 8.42 (telmisartan).

SPE recoveries of the 89 substances were determined at two pH values (pH 2 and pH 7) while the substances were spiked to deionized water before (10 ng/L) and after the SPE (10 µg/L). The mean recovery of SPE at pH 2 reached 62.1% ± 33.9% (± SD). Higher recoveries were achieved for the enrichment with pH 7, with 81.9% ± 27.9%. At the enrichment with pH 2, 21 substances revealed recoveries below 25%, where at pH 7, only 5 substances had recoveries below 25% (clopyralid, gabapentin, metoprolol acid, pregabalin, ritalinic acid), Table S 1. Four of the five substances with low recoveries at pH 7 also show a negative log K_{ow} value. This indicates polar functional groups of the substances, which can be less enriched by solid phase extraction than more non-polar substances. In order to check the repeatability of the SPE, the experiments were repeated once at both pH values, whereby a mean SD = 7.3% for pH 2 and a mean SD = 5.9% for pH 7 was achieved (Figure S 1). Due to the higher recovery, the SPE was carried out exclusively at pH 7 for the investigation of water samples.

The recovery of the extraction with the TLC-MS Interface was investigated in two approaches. In the first approach, the band length and peak width of the substances after the chromatography was not taken into account, considering only the dissolving capacity of the substances from the HPTLC plate. The mean recovery of the 89 substances reached 88.2% ± 7.2%, where three substances obtained recoveries below 25%: pindolol (2.4%), ciprofloxacin (16.3%) and oxytetracycline (12.4%). In the second approach, the recovery of the substances under the consideration of the mean peak width by separation with the gradient development in the AMD 2 (mean peak width: 1.9 mm) and with a band length of 6 mm was determined. Now the accurate positioning of the elution head was decisive for substance recovery. The mean recovery of the first approach amounted to 60.8% ± 9.5%, where additionally to the three substances in the second approach now ketoprofen (1.7%) and chlorobromuron (17.1%) also obtained recovery rates below 25%. The repeatability of the extraction (n = 5) in both approaches is shown in Figure S 2. These investigations revealed that the recovery strongly depends on the substances to be extracted. Furthermore, the accuracy of hitting the effective zone, equally leads to recovery losses as well as the solubility of the substances from the HPTLC plate.

The extraction recovery with the TLC-MS Interface is crucial for the developed workflow, because if the zone cannot be extracted from the plate or it is not properly hit by the elution head, the non-target evaluation and intersection formation cannot succeed and the prioritization fails. While losing the effective substance in the 1D is still noticeable because no effect can be detected in the 2D, losing it in the 2D remains unnoticed. In addition, low recovery of substances is further enhanced by double extraction of the substances from the HPTLC plate. To prevent the user from missing the zone to be extracted, an automated extraction would be helpful. Moreover, a flexible elution head that could be adapted to the peak width of the zone to be extracted could further reduce extraction losses.

Furthermore, it can be assumed that the recovery of the substances both during SPE and extraction from the HPTLC plate could be increased by specific method optimization, such as adjustments of the pH value or optimization of the extraction solvent. For example, substances with a carboxyl group could possibly be better recovered by lowering the pH value into the acidic range. In reality, however, the substances to be enriched are not known and several enrichments and extractions would have to be carried out in order to cover the different substance groups. This would lead to additional time and effort during the workflow and is therefore only recommended if information on the effective substances contained in the sample is already available.Finally, the total recovery of the workflow was calculated. Therefore, the extraction recoveries taking into account the geometry of the elution head and the band geometry were employed and recoveries above 100% (due to measurement fluctuations) were corrected to 100%. Considering the SPE enrichment at pH 2, the total recovery of the three extractions for the 89 substances was 24.6% ± 16.5%. Higher recoveries were achieved when applying data of the SPE enrichment at pH 7 with 31.7% ± 16.1%. The recoveries of the substances were determined using a mixed standard in methanol. When considering the recoveries in real water samples, even lower yields must be expected.

Due to these extraction losses, the concentration of the effective substances could decrease considerably up to the HRMS measurement. However, the detection limit of the AChE assay with approx. 1 µg/L lies significantly higher than the detection limit of the HPLC-HRMS detection (approx. 10 ng/L). Thus, also effective substances that are only partially transferred to the mass spectrometric detection may give a sufficient signal. Alternatively, the application volume to the HPTLC plate can be increased to compensate the extraction loss at the expense of sample extract.

The application of the heart-cut approach used in this study required an additional extraction step for the transfer of the effective zones from the 1D to the 2D. In comparison, with comprehensive 2D HPTLC separation and biotesting [21], the entire sample is separated in the 2D on the same plate and no extraction is required for the transfer from 1D to 2D. However, with this comprehensive 2D separation, only one sample can be separated per HPTLC plate and the bioassay can only be applied after the second separation step. Thus, the separation has to be repeated for the extraction of the effective zone after the 2D, because it is not possible to extract zones after the application of the bioassay. Since hR_F fluctuations are to be expected in both dimensions, the uncertainty of hitting the effective zone correctly becomes greater.

3.1.2. Proof of concept with spiked water samples

In order to check the developed workflow for its functionality, six acetylcholinesterase inhibiting substances were selected for spiking in water samples (Table 1). The spiked concentrations were determined on the basis of the strength of the substanceś effects. The selected substances covered diverse substance classes that cause inhibition through different bonds to the enzyme AChE. A further criterion for the substances was their hR_F in the 1D. The substances should be distributed over a broad retardation range, such that different MPs have to be applied in the 2D. To take into account different matrix influences, the workflow was carried out with drinking, surface and purified sewage water.

After the separation with the gradient development in the AMD 2 and effect detection with the AChE assay in the 1D (Fig. 2), the effective zones of the six spiked substances were extracted in all three samples. Dependent on their hR_F in the 1D (Table 1), the extracted zones were separated in the 2D with the MP A - D and the AChE assay was performed again (Figure S 3).

When comparing the hR_F of the spiked substances in the 1D and 2D (Fig. 3, A), it is noticeable that all the substances achieved different hR_F due to other selectivity and eluent strength of the used MPs. Also the hR_F order of the substances changed in the 2D. Substances with low hR_F in the 1D (galanthamine, donepezil and methiocarb-sulfoxide) migrated to higher hR_F in the 2D because of higher eluent strength (ε_{AB}) of the MP A and MP B (ε_{AB} = 0.65 resp. 0.56). The substances with higher hR_F in the 1D (caffeine,

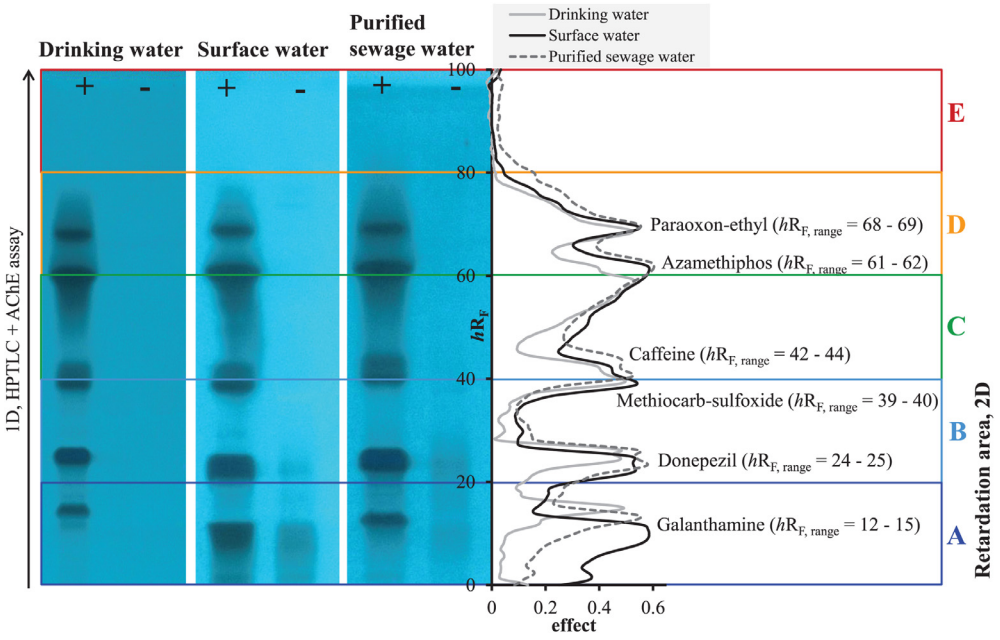


Fig. 2. AChE assay of the three spiked samples in the first dimension. +: spiked sample, - sample, A - E: hR_F area for the selection of the mobile phase A-E, which is used for the separation in the 2D.

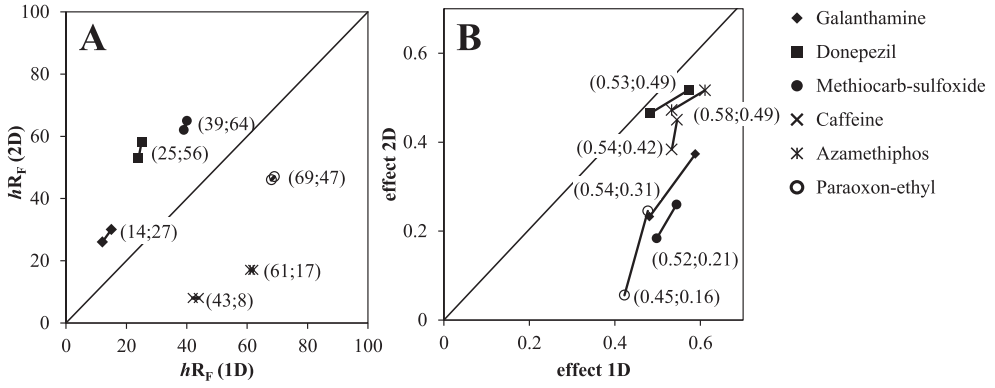


Fig. 3. hR_F (A) and effects (B) of the spiked substances in the 1D and 2D. The points indicate the range over the three spiked water samples.

azamethiphos and paraoxon-ethyl) migrated to lower hR_F in the 2D due to the lower eluent strength of the MP C and MP D ($\varepsilon_{AB} = 0.53$ resp. 0.40). A significant improvement of the separation was observed for the substances methiocarb-sulfoxide and caffeine. While these substances had almost identical hR_F in the 1D (hR_F (1D) = 39 and 43; $\alpha = 1.1$), they could be clearly separated in the 2D using the MP B and MP C (hR_F (2D) = 64 and 8; $\alpha = 8$).

In the 1D, the largest hR_F variation between the spiked samples occurred for the substance galanthamine ($hR_{F, range}$ = 12 - 15). The higher variation of the hR_F of galanthamine may be caused by matrix effects in the surface water. Diffuse effects to the AChE also occurred in the non-spiked surface water in the same hR_F range than galanthamine retardate (Fig. 2). This indicates an increased matrix load in this area, which may be responsible for the changed peak shape and the shift of galanthamine to lower hR_F in the surface water sample. The largest fluctuations of the hR_F occurred in the 2D by use of the eluent MP A and MP B (galanthamine $hR_{F, range}$ = 26 - 30; donepezil $hR_{F, range}$ = 53 - 58; methiocarb-sulfoxide $hR_{F, range}$ = 62 - 65).

When comparing the effects of the spiked substances in the 1D and 2D, all effects in the 2D are lower than in the 1D (Fig. 3, B). This can be explained by the recovery of the spiked substances during the extraction with the TLC-MS Interface, which reached 53.5% ± 5.2%. Nevertheless, a higher percentage of the substances effects were recovered, that was 64.1% ± 4.9%. Differing effect recoveries compared to substance recoveries can be explained by the dose-response relationship, which is not linear like the analytical signal of the substance concentration, but shows a sigmoidal course. Higher matrix content in the purified sewage and surface water compared to the drinking water showed no influence on the effect recovery.

Methiocarb-sulfoxide and caffeine each caused two effective zones in the 2D (Figure S 3). This can be traced back to the fact that these two substances migrate very close together in the 1D and for this reason cannot be extracted completely separate.

All the extracted zones of the 1D showed effects on the AChE assay in the 2D (Figure S 3) and were extracted again. The spiked sample extracts, the extracted zones of the 1D and the zones of the 2D were measured via HPLC-HRMS and the non-target evaluation and intersection formation was performed. For verification of the prioritization success, the number of signals in the sample extracts (without HPTLC separation) was compared with the number of signals in the 1D intersection (1D HPTLC separation) and in the 2D intersection (2D HPTLC separation), Table 2.

Table 2
Number of signals in the intersections of the spiked sample extracts, the extracted zones of the 1D and the extracted zones of the 2D, detected with HRMS (ESI+) for the proof of concept (spiked sample extract (1), extracted zone of the 1D (2), extracted zone of the 2D (3)).

Zone	Spiked substance	Drinking water			Surface water			Purified sewage water		
		1	1 ∩ 2	1 ∩ 2 ∩ 3	1	1 ∩ 2	1 ∩ 2 ∩ 3	1	1 ∩ 2	1 ∩ 2 ∩ 3
1	Galanthamine	339	21	18	4445	202	97	7816	82	26
2	Donepezil		30	17		174	28		305	44
3	Methiocarb-sulfoxide		10	10		59	32		109	37
4	Caffeine		10	2		53	14		89	25
5	Azamethiphos		62	28		71	52		104	49
6	Paraoxon-ethyl		43	8		46	22		80	27

As expected, the drinking water showed the lowest number of features in the sample extract (n = 339), compared to the surface water (n = 4445) and the purified sewage water (n = 7816). The number of features of all samples could be reduced through extraction of the effective zones from the HPTLC plate in the 1D and 2D. In any cases, the number of features in the intersection of sample extract, 1D and 2D plate extracts was lower than in the intersection of the sample extract and the 1D plate extract. That confirmed the usefulness of the second separation dimension. An exception was methiocarb-sulfoxide in drinking water, where the number of features in the 2D remained constant. Lowest signal numbers were always achieved in the drinking water and the purified sewage water contained the most signals in the intersections, with exception of the substances galanthamine and azamethiphos (Table 2).

In all three samples, the reduction in the number of signals in the 2D was more than 90%. It was shown that the filtering of relevant signals through 2D separation was most successful and most necessary for more complex samples. In the drinking water, the features were already adequately reduced after the 1D so that the 2D is of only little avail (mean signal quantity 1D: 31, 2D: 14). However, on average 137 signals were still present in the intersection of the purified sewage water after the 1D and the effort to look through every single signal for effective substances is very high. But only 35 ± 10 signals remained after the 2D separation, such that significant further reduction could be achieved. From this it can be concluded that for less polluted water with low expected overall effects, EDA with 1D HPTLC may be sufficient to filter relevant signals. However, if more complex samples, such as surface or sewage water should be examined, the EDA with 2D HPTLC approach is necessary.

The prerequisite for a successful prioritization is that the substances responsible for the effect are contained in the intersection at the end of the workflow and are not lost (false negative ones).

With the exception of methiocarb-sulfoxide in the drinking water, all the spiked substances were found in the 1D and 2D intersections of the three water samples. In the spiked drinking water, methiocarb-sulfoxide was transformed to methiocarb-sulfoxide phenol, which instead occurred in the extracted zone of the 1D. The loss of the substance in the intersection is therefore not a failure of the workflow, but rather a problem of stability of the effective substance. However, unstable substances must also be expected when examining real water samples. These can transform during the workflow and new signals are detected with the MS that are difficult to assign to the original bioactive substances.

In addition to the spiked substances, further signals remained in the intersections of the sample extracts. Some of them could be assigned to isotopologues and adducts of the spiked substances. So in all cases the sodium adducts of the spiked substances were detected and in several intersections also the dimers $[2M+H]^+$ and $[2M+Na]^+$. The automatic componentization for the evaluation of non-target data is not yet solved. Additional reaction products of some spiked substances, such as paraoxon-methyl and paraoxon-nitrophenylphosphate were detected, both resulting from reaction processes of paraoxon-ethyl.

When comparing the signal lists of the intersections, it was noticed that in most cases the spiked substances occurred with the highest signal intensity. Only the transformation product methiocarb-sulfoxide phenol was more intensive in the intersection of zone 3 in drinking water and surface water, than the precursor substance methiocarb-sulfoxide. In the intersection of zone 3 of the drinking water, the precursor methiocarb-sulfoxide was not detected at all, but only the transformation product. In the intersection of zone 5 of the drinking water, azamethiphos was only the fourth most intensive signal. The sodium adduct of azamethiphos was on the second place in the intersection, whereby the signals at the positions one and three could not be assigned to any molecular formula. Whereas the retention time of azamethiphos was 8.6 min, the retention times of the two unknown masses amounted to 6.1 min.

Finally, the recoveries of the spiked substances in the three water matrices were compared and the intensity losses of the substances during the workflow were determined. In all three water matrices, the intensities of the spiked substances decreased strongly during the workflow. After 1D extraction, the substances were recovered with 39.3% ± 1.3%, whereas the recovery in the 2D reached 25.3% ± 2.3%. Although the recovery of the substances was highest in the drinking water, the matrix of the water sample played a rather subordinate role for the recovery (mean recovery of the 1D in drinking water: 40.8%, surface water: 38.3%, purified sewage water: 38.8%). It was also shown that greater substance losses occurred in the 1D than in the 2D, which can be explained by the fact that the substance peaks were wider due to higher concentrations in 1D and could therefore only be extracted incompletely. Compared to the recoveries of the 89 substances (chapter 3.1.1), the values are in the same range and show that the recovery also depends strongly on the substances. For example, caffeine could be recovered with 42.3% ± 6.6% after the 2D, whereas the recovery of azamethiphos after the 2D was only 7.1% ± 5.9%. Despite the successful proof of concept, the intensity losses show the limitations of the workflow, such that substances with already low concentrations in the water samples may not be recovered after the 2D. Moreover, it is likely that samples with a high background of effects would have led to difficulties in localizing the effects of the spiked substances.

3.2. Investigation of real water samples

Three water samples were examined with the workflow, two surface waters and one untreated wastewater. After the 1D separation, many inhibiting effects on the AChE were detected (Fig. 4). Especially in the surface water A, the effective substances could not be separated completely and the potential neurotoxic effects were blended. Clearest effective zones could be detected in the untreated sewage water.

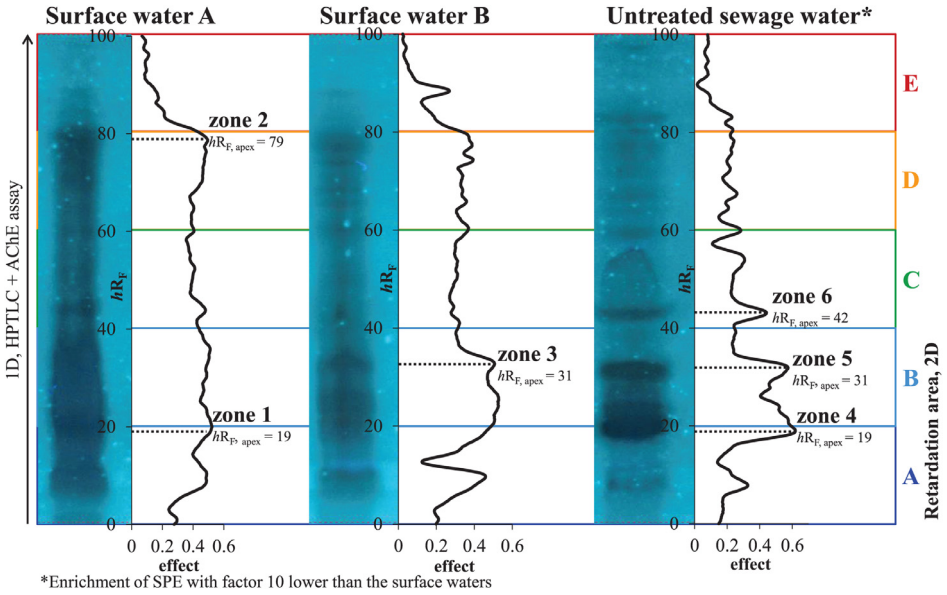


Fig. 4. AChE assay of three water samples in the 1D and selection of effective zones 1–6 for extraction, A - E: hR_F area for the selection of the mobile phase A-E, which is used for the separation in the 2D.

Six effective zones (zones 1 - 6) were selected for extraction with the zones being as intense and clear as possible. By extracting effective zones with the same hR_F in different samples, it should be checked whether the detected effects are possibly caused by the same substances.

The zones 1 - 5 showed clearly defined effects in the 2D, whereas with zone 6 no effect could be detected (Fig. 5). A weak effective area in the lower hR_F range suggests that the effect of the zone 6 in the 1D was caused by several rather weak effective substances, which were separated in the 2D. By observing the absorption scan at 220 nm and the fluorescence image at 366 nm, additionally to the effective zone also non-effective substances were detected, that could be separated from the effective zone through the second separation. For example, three fluorescent zones were detected in the zone 4, but none of them had any effect to the AChE. The hR_F of the zones 1 and 4 as well as of the zones 3 and 5, which already matched in the 1D, also matched after the second separation. Therewith the suspicion was confirmed that the same substances may be responsible for the detected effect. Additionally to the same hR_F in the 1D and 2D, also both the effective zones 3 and 5 showed a blue fluorescence at 366 nm. In all the cases, with exception of the zone 6, an absorption peak with the same hR_F than the effective zone could be detected, that may be caused by the effective substance.

With the exception of zone 6, the effects of all extracted zones were recovered in 2D. Only in zone 6 no clear effect could be detected. For this reason, no further extraction could be performed and the workflow was stopped for the zone 6.

The effective zones 1 - 5 of the 2D were extracted again and transferred to the HPLC-HRMS. Three samples per effect were measured: the sample extract, the extracted zone of the 1D and the extracted zone of the 2D. After the non-target evaluation, intersections of the detected signals were built (Table 3). Several thousand signals were detected in the surface water extracts A and B, whereas in the untreated waste water even about 18,000 signals occurred. By extracting the zones in the 1D, the number of remaining signals could be reduced to an average of 307. In the intersection of the 2D, only an average of 36 signals occurred.

The intersections of signals after the 2D were investigated to identify effective substances. Therefore, the signals were sorted

Table 3
Number of signals in the intersections of the water extracts, the extracted zones of the 1D and the extracted zones of the 2D, detected with HRMS ESI^+ (sample extract (1), extracted zone of the 1D (2), extracted zone of the 2D (3)).

Sample	Zone	1	1 ∩ 2	1 ∩ 2 ∩ 3
Surface water A	Zone 1	8681	462	28
Surface water A	Zone 2		68	3
Surface water B	Zone 3	7187	31	10
Untreated sewage water	Zone 4	17,721	540	33
Untreated sewage water	Zone 5		433	108

based on their intensity, starting with the most intense signals, which generally provide the most expressive isotope pattern and MS/MS spectra. In the first step, possible molecular formulas were determined on the basis of the MS, MS/MS and isotope pattern data (elements: C,H,O,N,S,P; Cl in the presence of a characteristic isotope pattern, mass tolerance: 10 ppm, intensity tolerance: 10 ppm). Structural formulas were searched in structural databases (Pubchem, ChemSpider and ForIdent). For confirmation of possible structures, the MS/MS data were compared with reference spectra in spectra databases (MassBank) and with *in-silico* fragmentation tools (MetFrag). When substances could be identified on the basis of their accurate masses, isotope pattern and MS/MS spectra, the structure was confirmed using a reference standard. With exception of the zones 2 and 6, possible effective substances could be identified in all the other zones. In zone 2, only three signals remained in the intersection of the 2D and none of them could be assigned to a structural formula, such that also the intersection after the 1D was investigated for candidates. But yet, no possible effective substance could be identified. All the substances identified via a reference standard (Level 1 and Level 2) are shown in Table 4.

The hR_F of the substances and their effects in the 1D and 2D were verified via the reference standards (Table 4, Figure S 4). Fluctuations in the hR_F may be explained by the separations on different HPTLC plates of samples and standards. In addition, the peak widths of the substances have to be considered. Even if the hR_F of a substance and the extracted zone are varying, the peak flank of the standard may overlap with the extraction area.

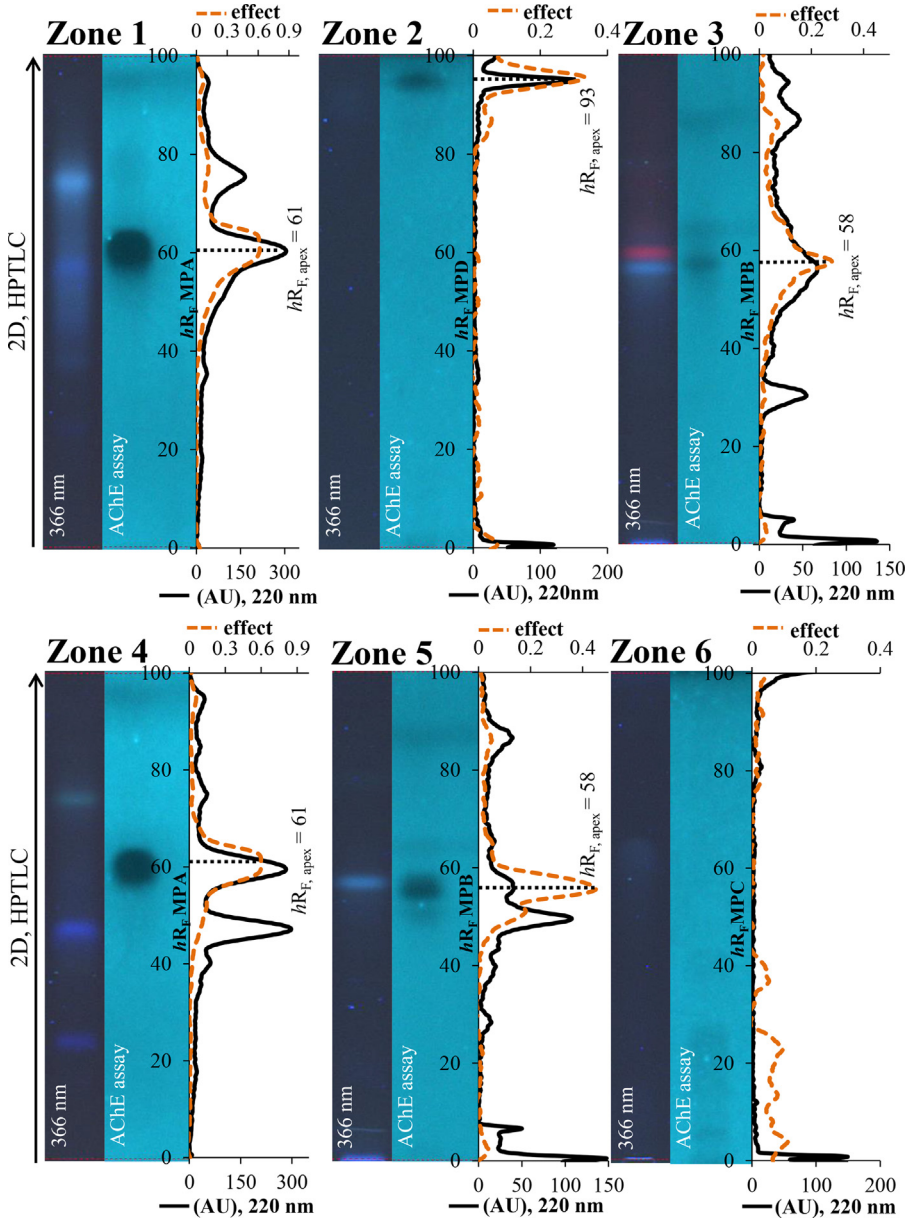


Fig. 5. Fluorescence image (366 nm), AChE assay (image and effect chromatogram) and absorption scan (220 nm) of extracted zones of the three water samples in the 2D.

Table 4
Identified substances in three water sample extracts via EDA with 2D HPTLC and HPLC-HRMS.

Sample	Zone	Substance	Molecular formula	hR_F 1D	hR_F 2D	*m/z*	RT / min	ß[1] / mg/L	ident. Level [28]
Surface water A	1	Clopidogrel CA[2]	$C_{15}H_{14}ClNO_2S$	20	62	308.0507	6.8	0.01	1
		Metolachlor ESA[3]	$C_{15}H_{23}NO_5S$	20	61	330.1370	8.0	0.3	1
		LAS C10-C13[4,5]	$C_nH_{2n+1}C_6H_4O_3S$ n = 10–13	19	63	-	-	28	2 1
		Triamterene	$C_{12}H_{11}N_7$	18	60	254.1146	5.7	0.01	
Surface water B	3	Lumichrome	$C_{12}H_{10}N_4O_2$	30	60	243.0877	7.2	0.2	1
		Methocarbamol	$C_{11}H_{15}NO_5$	31	56	242.1023	6.9	0.1	1
		Oxazepam	$C_{15}H_{11}ClN_2O_2$	32	59	287.0582	9.3	0.02	1
		Phenazone	$C_{11}H_{12}N_2O$	31	60	189.1022	6.5	0.03	1
Untreated sewage water	4	LAS C10-C13[4,5]	$C_nH_{2n+1}C_6H_4O_3S$ n = 10–13	19	63	–	–	97	2
Untreated sewage water	5	Lumichrome	$C_{12}H_{10}N_4O_2$	30	60	243.0877	7.2	0.3	1
		Paraxanthine	$C_7H_8N_4O_2$	31	56	181.0717	5.2	2.8	1

[1]Concentration in the enriched sample extract. Enrichment factor for surface water (zone 1 and zone 3): 1000, enrichment factor for untreated sewage water (zone 4 and zone 5): 100.
[2]Clopidogrel carboxylic acid
[3]Metolachlor ethanesulfonic acid
[4]Linear alkylbenzene sulfonates C10 - C13 homologues
[5]identified through ESI negative measurement, data not shown in this work.

The concentration of the identified substances was determined in the enriched sample extracts (Table 4), to be able to match the measured effect with the effect of the substances in these concentrations. For this reason, the concentration of the substances in the original water sample can only be estimated, since the recovery during the enrichment is not known. Moreover, it must be taken into account that there was only a reference standard for the substance sodium dodecylbenzene sulfonate that contained a mixture of linear alkylbenzene sulfonate (LAS) homologues (C10 - C13), each with different isomers. While the homologues could still be separated partly in the HPLC, the isomers overlapped, so that only a semi-quantitative estimation of the LAS concentration could be performed.

All the substance concentrations ranged from 10 µg/L to a maximum of 2.75 mg/L in the enriched samples. The exceptions were the LAS, which were found in the samples extracts (surface water A and untreated sewage water) with concentrations in the mean mg/L area (semi-quantitative estimation). By mixing the identified substances in a zone in the quantified concentrations, separating them in the 1D and performing the AChE assay, the proportion of the explainable effect could be determined.

In the surface water A at $hR_F = 19$ (zone 1), the potential neurotoxic effect could be fully explained by the mix of the four identified substances ($effect_{mix\ 1}/effect_{zone\ 1}$: 1.05). By investigation of the four substances individually with the AChE assay, only the LAS homologues (C10 - C13) caused an effect on the AChE. Since the homologues and isomers of the LAS group on the HPTLC plate could not be separated from each other, it could not be conclusively determined whether the effect was caused by one or all homologues or isomers in combination. The other three substances in mix 1 (clopidogrel CA, metolachlor ESA and triamterene) showed no effect, even at higher concentrations (100 mg/L). The effect at $hR_F = 19$ in the untreated sewage water could also be explained almost completely by the LAS concentration ($effect_{LAS}/effect_{zone\ 4}$: 0.90). For the zones 3 and 5, however, only a small part of the effects in the samples could be explained with the identified substances ($effect_{mix\ 3}/effect_{zone\ 3}$: 0.07; $effect_{mix\ 5}/effect_{zone\ 5}$: 0.14). In the mix 3 (lumichrome, methocarbamol, oxazepam and phenazon), only the substance lumichrome inhibited the enzyme AChE, whereas the other three substances showed no effect. In the mix 5, however, both the substances lumichrome and paraxanthine inhibited the AChE and so together caused the effect in the untreated sewage water at $hR_F = 31$. The proportion of the effects that cannot be explained with the identified substances may be caused by further unknown substances also contributing to the effects. This finding is similar to results of other studies, where also only parts of the detected effects could be explained [13,29,30]. It has to be considered that some bioactive substances are not detectable with LC-HRMS at all, such that the used workflow cannot succeed in these cases. Additionally, many more effects in the investigated samples detected in the 1D bioassay remained unknown, because due to time constraints only a certain number of strong effective zones can be selected for identification. This demonstrated the difficulty of the identification, which despite all the progress made in separation efficiency and prioritization still poses a great challenge.

Especially for such heavily loaded samples, other multi-dimensional separations could also be suitable for time reasons. Instead of selective 2D HPTLC separation, it would also be possible to use comprehensive 2D HPTLC separations, which have the advantage that no individual fractions have to be selected. In the case of highly blurred effects that were distributed over the entire hR_F range, laborious extraction of zones over the entire range or the difficult selection of individual effective zones would be eliminated and instead the entire sample would be separated in 2D. Another opportunity would be multi-dimensional HPLC separation of the extracted effective zone. This could possibly lead to higher separation efficiency with lower time expense. In addition, different mobile phases may be used for HPTLC separation of one sample. By extracting the effective zone after each separation and measuring them with HPLC-HRMS, the intersection of the signals occurring in all the extracted zones could be determined. Maybe the success of identification would be increased with such applications.

However, for the suitability of multi-dimensional separations for routine applications, the time and effort required must be taken into account. More separations and more extractions increase the experimental time, such that they are only conditionally suitable for the application with many samples. Rather, the application of such multi-dimensional separations, as also used in our developed workflow lies in the examination of conspicuous samples, where 1D separation does not lead to successful identification of effective compounds.The effective substances identified in this work differ greatly in their areas of application. Lumichrome is a derivate of riboflavin and is created by its photolysis. As a plant metabolite, it is crucial for the growth of plants [31]. Lumichrome was already found as AChE inhibiting substance *in-vitro* with an IC_{50} of 12.24 µM ± 0.12 µM and was comparable to the effect of galantamine (IC_{50} = 11.31 µM ± 0.11 µM) in this study [32]. Paraxanthin is a derivate of caffeine that is used in many ways as a stimulant. The AChE inhibiting effect of paraxanthin has already been described in earlier studies [33]. The effective substance of the LAS group could not be clearly identified (Level 2 identification), because the homologues and isomers contained in the available reference standard could not be separated in both dimensions by HPTLC. For clear identification of the effective substance(s), sufficiently pure reference standards of the substances would have to be available. For these reasons, the concentration of LAS in the two water samples could so far only be estimated semi-quantitatively. Linear alkylbenzene sulfonates are used in large quantities in detergents and cleaning agents and are released into the environment by incomplete degradation in sewage water treatment plants [34]. The AChE inhibiting effect of LAS has already been described in previous studies. For example, in a work of Guilhermino, inhibitory effects of dodecyl benzyl sulfonate (DBS) and sodium dodecyl sulfate (SDS) on the AChE of Daphnia magna were demonstrated *in-vivo* and *in-vitro* in the mg/L range. It must be taken into account that LAS do not inhibit the enzyme by a specific binding to the active center, but cause a non-specific inhibition [35].

To finally asses the detected effects, further bioassays should be performed in addition to the AChE assay. Since the AChE assay is an initial assay for the detection of potential neurotoxic effects, its results have to be verified by further bioassays gathering neurotoxicity. Thus, *in-vivo* assays also considering toxicokinetic processes should be applied. However, such bioassays often require a great deal of effort and long experimental periods, which is why simple and fast assays are often used to get a general overview of effects that are then confirmed by such elaborate assays.

4. Conclusions

In this study, a workflow to prioritize effective compounds with EDA with 2D HPTLC in combination to HPLC-HRMS was presented. Although the workflow is elaborate and involves manual steps, the present study reveals its efficiency in prioritizing of signals for the identification of effective substances. Investigating samples that contain many unknown compounds, a single extraction of the effective zones from the HPTLC plate is not sufficient to reduce the number of signals to a manageable quantity. Therefore, the EDA with 2D HPTLC is required.

Through application of the developed workflow to water samples, several substances could be identified successfully. However, in some cases substances have been identified, which have no

effect in the bioassay. More available literature data, containing toxicity information of the substances may facilitate the identification of effective substances. Despite the successful identification of some effective substances, a large number of effects still remain unknown in the investigated samples, since the effort required to identify only one substance is immense.

The EDA with 2D HPTLC workflow was performed with the AChE assay for the detection of potential neurotoxic effects in this study. However, it could be easily adapted to further bioassays in the future, such that a wider effect spectrum may be covered.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

CRediT authorship contribution statement

Lena Stütz: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing - original draft. **Wolfgang Schulz:** Conceptualization, Methodology, Formal analysis, Writing - review & editing, Supervision. **Rudi Winzenbacher:** Conceptualization, Resources, Writing - review & editing, Supervision.

Acknowledgements

The authors thank the German Federal Ministry for Education and Research (BMBF) for the funding and the financial support of the project NeuroBox under the grant 02WRS1419E.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2020.461239.

References

[1] , Transforming Our World: the 2030 Agenda for Sustainable Development (A/RES/70/1), United Nations, 2015.
[2] W. Busch, S. Schmidt, R. Kühne, et al., Micropollutants in European rivers: a mode of action survey to support the development of effect-based tools for water monitoring, Environ. Toxicol. Chem. 35 (2016) 1887–1899 https://doi.org/10.1002/etc.3460.
[3] S.D. Richardson, T.A. Ternes, Water analysis: emerging contaminants and current issues, Anal. Chem. 86 (2014) 2813–2848 https://doi.org/10.1021/ac500508t.
[4] N. Hermes, K.S. Jewell, A. Wick, et al., Quantification of more than 150 micropollutants including transformation products in aqueous samples by liquid chromatography-tandem mass spectrometry using scheduled multiple reaction monitoring, J. Chromatogr. A 1531 (2018) 64–73 https://doi.org/10.1016/j.chroma.2017.11.020.
[5] Y. Peng, W. Fang, M. Krauss, et al., Screening hundreds of emerging organic pollutants (EOPs) in surface water from the Yangtze River Delta (YRD): occurrence, distribution, ecological risk, Environ. Pollut. 241 (2018) 484–493 https://doi.org/10.1016/j.envpol.2018.05.061.
[6] R. Altenburger, S. Ait-Aissa, P. Antczak, et al., Future water quality monitoring - adapting tools to deal with mixtures of pollutants in water resource management, Sci. Total Environ. 512–513 (2015) 540–551 https://doi.org/10.1016/j.scitotenv.2014.12.057.
[7] J. Hollender, E.L. Schymanski, H.P. Singer, et al., Nontarget screening with high resolution mass spectrometry in the environment: ready to go? Environ. Sci. Technol. 51 (2017) 11505–11512 https://doi.org/10.1021/acs.est.7b02184.
[8] E.L. Schymanski, H.P. Singer, J. Slobodnik, et al., Non-target screening with high-resolution mass spectrometry: critical review using a collaborative trial on water analysis, Anal. Bioanal. Chem. 407 (2015) 6237–6255 https://doi.org/10.1007/s00216-015-8681-7.
[9] W. Brack, V. Dulio, M. Ågerstrand, et al., Towards the review of the European Union Water Framework management of chemical contamination in European surface water resources, Sci. Total Environ. 576 (2017) 720–737 https://doi.org/10.1016/j.scitotenv.2016.10.104.
[10] W. Brack, S. Aït-Aïssa, R.M. Burgess, et al., Effect-directed analysis supporting monitoring of aquatic environments - an in-depth overview, Sci. Total Environ. 544 (2016) 1073–1118 https://doi.org/10.1016/j.scitotenv.2015.11.102.
[11] M. Hecker, H. Hollert, Effect-directed analysis (EDA) in aquatic ecotoxicology: state of the art and future challenges, Environ. Sci. Pollut. Res. 16 (2009) 607–613 https://doi.org/10.1007/s11356-009-0229-y.
[12] E.L. Schymanski, M. Bataineh, K.U. Goss, et al., Integrated analytical and computer tools for structure elucidation in effect-directed analysis, TrAC, Trends Anal. Chem. 28 (2009) 550–561 https://doi.org/10.1016/j.trac.2009.03.001.
[13] F. Itzel, K.S. Jewell, J. Leonhardt, et al., Comprehensive analysis of antagonistic endocrine activity during ozone treatment of hospital wastewater, Sci. Total Environ. 624 (2018) 1443–1454 https://doi.org/10.1016/j.scitotenv.2017.12.181.
[14] X. Ouyang, P.E.G. Leonards, Z. Tousova, et al., Rapid screening of acetylcholinesterase inhibitors by effect-directed analysis using LC × LC fractionation, a high throughput in vitro assay, and parallel identification by time of flight mass spectrometry, Anal. Chem. 88 (2016) 2353–2360 https://doi.org/10.1021/acs.analchem.5b04311.
[15] W. Brack, Effect-directed analysis: a promising tool for the identification of organic toxicants in complex mixtures? Anal. Bioanal. Chem. 377 (2003) 397–407 https://doi.org/10.1007/s00216-003-2139-z.
[16] G. Morlock, W. Schwack, Hyphenations in planar chromatography, J. Chromatogr. A 1217 (2010) 6600–6609 https://doi.org/10.1016/j.chroma.2010.04.058.
[17] S.C. Weiss, N. Egetenmeyer, W. Schulz, In vitro Environmental Toxicology - Concepts, Application and Assessment, "Coupling of In Vitro Bioassays with Planar Chromatography in Effect-Directed Analysis, Springer, Berlin, Heidelberg, 2016, pp. 187–224 https://doi.org/10.1007/10_2016_16.
[18] Á.M. Móricz, P.G. Ott, T.T. Häbe, et al., Effect-directed discovery of bioactive compounds followed by highly targeted characterization, isolation and identification, exemplarily shown for Solidago virgaurea, Anal. Chem. 88 (2016) 8202–8209 https://doi.org/10.1021/acs.analchem.6b02007.
[19] L. Stütz, S.C. Weiss, W. Schulz, et al., Selective two-dimensional effect-directed analysis with thin-layer chromatography, J. Chromatogr. A 1524 (2017) 273–282 https://doi.org/10.1016/j.chroma.2017.10.009.
[20] T. Mroczek, Qualitative and quantitative two-dimensional thin-layer chromatography/high performance liquid chromatography/diode-array/electrospray-ionization-time-of-flight mass spectrometry of cholinesterase inhibitors, J. Pharm. Biomed. Anal. 129 (2016) 155–162 https://doi.org/10.1016/j.jpba.2016.06.048.
[21] B. Milz, B. Spangenberg, Grafted 2D-thin layer chromatography (2D-TLC) of 17α-ethinylestradiol detected by bio-directed analysis, J. Liq. Chromatogr. Relat. Technol. 37 (2014) 2872–2881 https://doi.org/10.1080/10826076.2014.907109.
[22] H. Li, J. Zhang, J. You, Diagnosis of complex mixture toxicity in sediments: application of toxicity identification evaluation (TIE) and effect-directed analysis (EDA), Environ. Pollut. 237 (2018) 944–954 https://doi.org/10.1016/j.envpol.2017.11.005.
[23] P.A. Neale, W. Brack, S. Aït-Aïssa, et al., Solid-phase extraction as sample preparation of water samples for cell-based and other in vitro bioassays, Environ. Sci.: Processes Impacts 20 (2018) 493–504 https://doi.org/10.1039/C7EM00555E.
[24] G.E. Morlock, Background Mass Signals in TLC/HPTLC–ESI-MS and Practical Advices for use of the TLC-MS Interface, J. Liq. Chromatogr. Relat. Technol. 37 (2014) 2892–2914 https://doi.org/10.1080/10739149.2014.907000.
[25] T. Bader, W. Schulz, K. Kümmerer, et al., General strategies to increase the repeatability in non-target screening by liquid chromatography-high resolution mass spectrometry, Anal. Chim. Acta 935 (2016) 173–186 https://doi.org/10.1016/j.aca.2016.06.030.
[26] T. Bader, W. Schulz, K. Kümmerer, et al., LC-HRMS data processing strategy for reliable sample comparison exemplified by the assessment of water treatment processes, Anal. Chem. 89 (2017) 13219–13226 https://doi.org/10.1021/acs.analchem.7b03037.
[27] N. Zwart, S.L. Nio, C.J. Houtman, et al., High-throughput effect-directed analysis using downscaled in vitro reporter gene assays to identify endocrine disruptors in surface water, Environ. Sci. Technol. 52 (2018) 4367–4377 https://doi.org/10.1021/acs.est.7b06604.
[28] E.L. Schymanski, J. Jeon, R. Gulde, et al., Identifying small molecules via high resolution mass spectrometry: communicating confidence, Environ. Sci. Technol. 48 (2014) 2097–2098 https://doi.org/10.1021/es5002105.
[29] P.A. Neale, N.A. Munz, S. Aït-Aïssa, et al., Integrating chemical analysis and bioanalysis to evaluate the contribution of wastewater effluent on the micropollutant burden in small streams, Sci. Total Environ. 576 (2017) 785–795 https://doi.org/10.1016/j.scitotenv.2016.10.141.
[30] P.A. Neale, R. Altenburger, S. Aït-Aïssa, et al., Development of a bioanalytical test battery for water quality monitoring: fingerprinting identified micropollutants and their contribution to effects in surface water, Water Res 123 (2017) 734–750 https://doi.org/10.1016/j.watres.2017.07.016.
[31] F. Dakora, V. Matiru, A. Kanu, Rhizosphere ecology of lumichrome and riboflavin, two bacterial signal molecules eliciting developmental changes in plants, Front. Plant Sci. 6 (2015) 1–11 https://doi.org/10.3389/fpls.2015.00700.
[32] J.S.A. Alves, A.J. Pereira, T. Dethoup, et al., A new meroterpene, a new benzofuran derivative and other constituents from cultures of the marine sponge-associated fungus acremonium persicinum KUFA 1007 and their anticholinesterase activities, Mar. Drugs 17 (2019) 1–13 https://doi.org/10.3390/md17060379.
[33] M. Pohanka, The effects of caffeine on the cholinergic system, Mini-Rev. Med. Chem. 14 (2014) 543–549, doi:10.2174/1389557514666140529223436.

[34] M. Scheurer, F. Freeling, N.A. Alygizakis, et al., Development of an analytical method for the quantification of surfactants and its application to wastewater treatment plant effluents, Umweltbundeamt (2019) http://www.umweltbundesamt.de/publikationen.

[35] L. Guilhermino, M.N. Lacerda, A.J.A. Nogueira, et al., In vitro and in vivo inhibition of Daphnia magna acetylcholinesterase by surfactant agents: possible implications for contamination biomonitoring, Sci. Total Environ. 247 (2000) 137–141 https://doi.org/10.1016/S0048-9697(99)00485-4.

Supplementary material

Identification of acetylcholinesterase inhibitors in water by combining two-dimensional thin-layer chromatography and high-resolution mass spectrometry

Lena Stütz[a,b*], Wolfgang Schulz[a], Rudi Winzenbacher[a]

[a]Laboratory for Operation Control and Research, Zweckverband Landeswasserversorgung, Am Spitzigen Berg 1, 89129 Langenau, Germany

[b]Institute of Food Chemistry, University of Hohenheim, Garbenstraße 28, 70599 Stuttgart, Germany

E-mail addresses:
Stuetz.L@lw-online.de
Schulz.W@lw-online.de
Winzenbacher.R@lw-online.de

*Corresponding author:
Lena Stütz
E-mail address: Stuetz.L@lw-online.de
Tel.: +49 (7345) 9638-2226

Table of contents

List of Tables

List of Figures

Table S 1: Substance recoveries of SPE (N = 2) and extraction with the TLC-MS Interface (N = 5), the total recovery of the developed workflow and the log K_{ow}-values

Substance	Recovery SPE pH 2, in %	Recovery SPE pH 7, in %	Recovery extraction A[1], in %	Recovery extraction B[2], %	Recovery workflow, pH 2, in %	Recovery workflow, pH 7, in %	$logK_{ow}$ (EPIWEB 4.1)
2-Phenyl-2-ethylmalonamide	77.3	56.5	101.1	71.4	39.4	28.8	0.40
Acebutolol	76.6	88.7	72.5	57.2	25.0	29.0	1.19
Acetaminophen	22.4	87.5	86.4	28.6	1.8	7.2	0.27
Acetylsulfamethoxazol	98.4	103.2	97.7	48.2	22.9	23.2	1.21
Alprenolol	94.0	105.0	85.4	65.7	40.6	43.2	2.81
Amantadin	3.4	52.8	44.0	39.5	0.5	8.3	2.43
Amisulpride	87.6	84.5	58.3	50.7	22.5	21.7	1.11
Amisulpride N-Oxide	86.4	97.7	85.6	62.5	33.8	38.2	0.35
Atenolol	2.6	27.1	59.6	58.1	0.9	9.1	-0.03
Azithromycin	74.3	59.5	76.4	77.8	45.0	36.0	3.24
Bentazone	87.9	98.8	105.8	45.0	17.8	20.0	1.67
Betaxolol	99.0	105.2	84.8	65.7	42.7	43.2	2.98
Bezafibrate	94.5	100.0	98.4	66.4	41.7	44.1	4.25
Bisoprolol	86.3	104.8	82.3	65.8	37.3	43.3	1.84
Bisoprolol M4	15.8	86.1	75.1	61.2	5.9	32.3	0.64
Bromacil	92.1	110.8	99.3	67.9	42.4	46.1	1.68
Carbamazepin-10,11-epoxide	38.8	103.5	100.1	58.8	13.4	34.5	0.95
Carbamazepine	94.7	101.4	100.3	67.6	43.3	45.7	2.25
Chloramben	83.9	46.2	94.1	62.0	32.3	17.8	1.90
Chloramphenicol	86.4	132.2	105.0	66.2	37.9	43.9	0.92
Chlorbromuron	89.4	97.9	93.0	17.1	2.6	2.8	3.15
Ciprofloxacin	83.8	81.6	16.3	9.2	0.7	0.7	0.00
Clarithromycin	40.8	77.7	83.0	76.9	24.1	45.9	3.18
Clenbuterol	79.0	94.5	88.8	68.3	36.9	44.1	2.00
Clindamycin	14.1	85.3	81.2	69.8	6.8	41.5	2.01
Clopyralid	99.0	19.2	89.9	63.8	40.3	7.8	1.63
Codeine	11.5	85.2	76.3	67.8	5.3	39.2	1.28
Crotamiton	11.7	88.5	97.4	64.2	4.8	36.5	2.73
Dapsone	38.6	101.9	94.9	57.3	12.7	32.8	0.77
Desamino-metamitron	104.3	97.1	101.4	73.5	54.0	52.5	1.43
Diazepam	98.2	101.1	99.6	70.9	49.4	50.3	2.70
Diclofenac	90.0	95.9	90.9	41.4	15.4	16.4	4.02
Dihydrocodeine	9.3	89.0	67.7	57.3	3.0	29.2	1.49
Dimethenamid M27	87.0	109.7	85.7	56.3	27.6	31.7	-
Erythromycin	18.9	56.3	86.7	73.9	10.3	30.7	2.48

Substance	Recovery SPE pH 2, in %	Recovery SPE pH 7, in %	Recovery extraction A[1], in %	Recovery extraction B[2], %	Recovery workflow, pH 2, in %	Recovery workflow, pH 7, in %	$logK_{ow}$ (EPIWEB 4.1)
Etofibrat	65.5	50.0	96.9	57.6	21.7	16.6	3.43
Fenofibrate	75.4	82.3	72.3	66.8	33.6	36.7	5.19
Fenoxaprop	11.6	93.6	96.8	64.0	4.7	38.3	4.17
Flecainide	96.3	92.9	93.8	71.9	49.8	48.0	3.95
Fluazifop	96.1	107.4	98.0	71.8	49.6	51.6	3.58
Fluroxypyr	86.5	108.5	96.2	67.4	39.3	45.4	1.17
Gabapentin	2.9	4.1	100.0	68.5	1.3	1.9	-1.37
Gabapentin-Lactam	57.1	89.4	100.7	71.7	29.3	45.9	1.91
Haloxyfop(-P) free acid	87.7	101.9	97.9	66.5	38.8	44.2	3.38
Hydrochlorothiazide	92.4	102.4	84.6	66.8	41.2	44.6	-0.10
Indomethacin	68.1	92.4	91.4	62.4	26.5	36.0	1.43
Ketoprofen	85.5	111.6	67.5	1.7	0.0	0.0	3.00
Lamotrigine	45.9	91.5	101.7	75.0	25.9	51.5	0.99
Lamotrigin-N2-Oxide	58.1	78.7	94.9	68.9	27.6	37.4	2.91
Metconazole	91.2	103.3	95.7	74.5	50.7	55.5	4.19
Methylphenidate hydro-chloride C II	43.6	88.2	102.2	72.8	23.1	46.8	2.78
Metoprolol	93.0	90.1	79.8	65.2	39.5	38.3	1.69
Metoprolol acid	11.6	4.1	95.7	66.2	5.1	1.8	-2.34
Metronidazol	92.9	33.5	99.2	71.7	47.7	17.2	0.00
N,N-Diethyltoluamide	21.0	87.4	98.1	68.0	9.7	40.5	2.26
N-Acetyl-4-aminoantipyrin	82.4	57.2	101.4	41.3	14.1	9.8	-0.13
Naproxen	93.5	109.5	115.0	73.0	49.8	53.2	3.10
N-Formyl-4-aminoantipyrin	57.8	97.6	103.1	41.1	9.8	16.5	0.50
NMP	14.2	33.5	79.0	58.5	4.9	11.5	-0.11
Oxazepam	88.3	104.7	97.3	65.1	37.5	42.4	2.32
Oxytetracycline	9.0	28.7	12.4	11.6	0.1	0.4	-1.72
Pentoxifylline	74.7	98.9	85.6	69.1	35.6	47.2	0.56
Phenacetin	91.7	100.3	99.8	69.6	44.4	48.4	1.67
Phenazone	87.6	91.1	98.7	57.6	29.0	30.2	0.59
Phenylalanine	30.8	91.9	89.1	61.2	11.6	34.5	2.89
Picloram	93.2	45.5	91.1	59.5	33.0	16.1	1.36
Pindolol	4.1	58.4	2.4	10.0	0.0	0.6	1.48
Pregabalin	0.5	0.4	94.4	65.1	0.2	0.2	-1.78
Primidone	94.3	99.5	102.5	72.1	49.1	51.8	0.73
Propranolol	87.2	100.0	78.4	60.3	31.7	36.3	2.60
Propyphenazone	78.6	90.4	98.3	54.3	23.2	26.7	2.05

Substance	Recovery SPE pH 2, in %	Recovery SPE pH 7, in %	Recovery extraction A[1], in %	Recovery extraction B[2], %	Recovery workflow, pH 2, in %	Recovery workflow, pH 7, in %	$logK_{ow}$ (EPIWEB 4.1)
Quinmerac	93.6	61.9	94.1	66.2	41.0	27.1	2.87
Ritalinic acid	19.6	11.8	101.6	72.6	10.3	6.2	-1.07
Ronidazol	85.2	98.1	98.6	70.3	42.1	48.5	-0.37
Roxithromycin	38.0	63.1	77.4	81.8	25.4	42.2	2.75
Sitagliptin	33.2	62.5	90.9	68.8	15.7	29.6	1.39
Sotalol	0.0	51.6	76.1	63.8	0.0	21.0	0.37
Sulfadiazine	70.8	92.4	97.4	54.6	21.1	27.5	-0.34
Sulfadimidin	73.6	88.8	96.2	52.2	20.0	24.2	0.76
Sulfamerazine	70.4	95.6	97.9	49.6	17.3	23.5	0.21
Sulfamethoxazole	64.8	107.1	91.4	34.2	7.6	11.7	0.48
Sulfathiazol	47.3	90.4	99.2	72.8	25.0	47.9	0.72
Sulpirid	6.4	94.2	69.4	65.3	2.7	40.1	0.65
Sulpiride N-Oxide	11.5	42.9	90.0	76.7	6.8	25.2	-0.12
Tramadol	53.8	91.8	96.1	69.8	26.2	44.8	3.05
Tramadol N-Oxide	41.7	94.3	102.3	73.6	22.6	51.0	2.24
trans-10,11-Dihydroxy-10,11-dihydrocarbamazepin	115.4	96.1	102.1	64.2	41.2	39.6	-0.21
Triclopyr	99.0	105.1	93.3	72.9	52.6	53.2	2.53
Trimethoprim	53.6	92.1	101.7	76.5	31.4	53.9	0.73
mean	62.1	81.9	88.2	60.8	24.6	31.7	1.5
SD	33.9	27.9	18.9	16.0	16.5	16.1	1.5

[1]without considering the geometry of the elution head and the band geometry

[2]consideration of the geometry of the elution head and the band geometry

Table S 2: Composition of the used gradient development in the AMD 2

Step	Methanol:formic acid (100:0.05, v/v (%)	Dichloromethane (%)	n-Hexane (%)	Migration distance (mm)
1	100.0	0.0	0.0	11.0
2	100.0	0.0	0.0	11.0
3	100.0	0.0	0.0	11.0
4	70.0	30.0	0.0	16.3
5	30.0	70.0	0.0	21.6
6	20.0	80.0	0.0	26.9
7	15.0	85.0	0.0	32.2
8	11.0	89.0	0.0	37.5
9	8.5	91.5	0.0	42.8
10	6.5	93.5	0.0	48.2
11	5.0	95.0	0.0	53.5
12	4.0	96.0	0.0	58.8
13	3.5	96.5	0.0	64.1
14	0.0	100.0	0.0	69.4
15	0.0	50.0	50.0	74.7
16	0.0	0.0	100.0	80.0

Table S 3: Mobile phases for separation in the 2D

No.	hR_F area	Composition MP	Steps	Migration distance (mm)	Eluent strength ε_{AB}	Separation apparatus
A	[0, 20]	Formic acid-methanol-chloroform (3.3:36.6:60, v/v/v)	1	70	0.65	ADC
B	]20, 40]	Methanol-chloroform (20:80, v/v)	1	70	0.56	ADC
C	]40, 60]	Ethyl acetate-dichloromethane-n-hexane (65:10:25, v/v/v)	2	70	0.53	AMD
D	]60, 80]	Acetonitrile-chloroform-n-hexane (7:70:23, v/v/v)	3	70	0.40	AMD
E	]80, 100]	Toluene-n-hexane (70:30, v/v)	2	70	0.23	AMD

Table S 4: Stability of hR_F values of the chromatography control mix

Substance	Mobile phases mean hR_F ± SD				
	Gradient development (N = 10)	**MP A (N = 5)**	**MP B (N = 5)**	**MP C (N = 3)**	**MP D (N = 3)**
1,3,6-Naphthalenesulfonic acid	11.3 ± 0.7	-	-	-	-
1,5-Naphthalenesulfonic acid	13.2 ± 0.4	10.7 ± 1.2	0.3 ± 0.6	-	-
1-Naphthalenesulfonic acid	18.7 ± 0.9	48.0 ± 1.7	6.0 ± 1.0	-	-
Theobromine	27.8 ± 0.8	73.0 ± 4.0	50.3 ± 1.2	2.3 ± 0.6	-
Caffeine	30.9 ± 0.8	-	64.7 ± 1.5	13.0 ± 1.0	-
Thiourea	41.9 ± 1.3	62.3 ± 2.3	30.3 ± 2.5	6.7 ± 0.6	-
N-Phenylacetamide	56.0 ± 1.0	-	60.0 ± 1.0	66.3 ± 0.6	22.3 ± 1.5
Benzamide	75.9 ± 0.9	94.0 ± 3.6	76.0 ± 1.0	-	77.7 ± 2.1
N,N-Dimethyl-4-[(E)-phenyldiazenyl]-aniline	82.6 ± 0.5	97.7 ± 2.5	86.0 ± 1.0	-	-

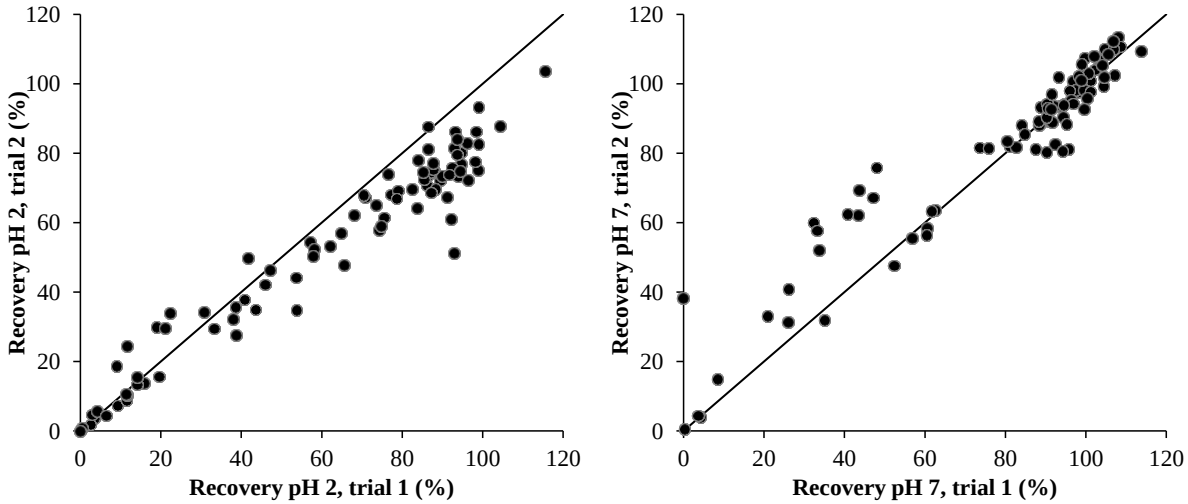
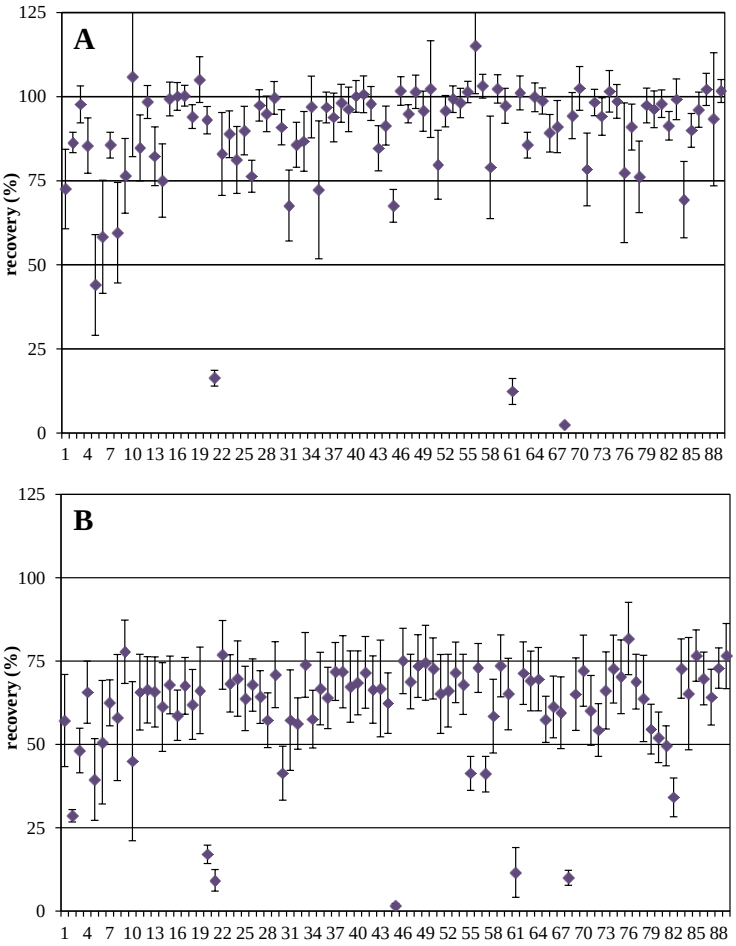


Figure S 1: Repeatability of solid phase extraction with pH 2 respectively pH 7



1	Acebutolol	46	Lamotrigine
2	Acetaminophen	47	Lamotrigin N2-Oxid
3	Acetylsulfamethoxazol	48	Metamitron
4	Alprenolol	49	Metconazole
5	Amantadin	50	Methylphenidate hydrochloride C II
6	Amisulprid	51	Metoprolol
7	Amisulpride N-Oxide	52	Metoprololic acid
8	Atenolol	53	Metronidazol
9	Azithromycin	54	N,N-Diethyltoluamide (DEET)
10	Bentazone	55	N-Acetyl-4-aminoantipyrin
11	Betaxolol	56	Naproxen
12	Bezafibrate	57	N-Formyl-4-aminoantipyrin
13	Bisoprolol	58	NMP
14	Bisoprolol_M4	59	N-Oxide Tramadol
15	Bromacil	60	Oxazepam
16	Carbamazepin-10,11-epoxid	61	Oxytetracycline
17	Carbamazepine	62	PEMA (2-Phenyl-2-ethylmalonamid)
18	Chloramben	63	Pentoxifylline
19	Chloramphenicol	64	Phenacetin
20	Chlorbromuron	65	Phenazone
21	Ciprofloxacin	66	Phenylalanine
22	Clarithromycin	67	Picloram
23	Clenbuterol	68	Pindolol
24	Clindamycin	69	Pregabalin
25	Clopyralid	70	Primidone
26	Codeine	71	Propranolol
27	Crotamiton	72	Propyphenazone
28	Dapsone	73	Quinmerac
29	Diazepam	74	Ritalinic acid
30	Diclofenac	75	Ronidazol
31	Dihydrocodeine	76	Roxithromycin
32	Dimethenamid M27	77	Sitagliptin
33	Erythromycin	78	Sotalol
34	Etofibrat	79	Sulfadiazine
35	Fenofibrate	80	Sulfadimidin
36	Fenoxaprop	81	Sulfamerazine
37	Flecainide	82	Sulfamethoxazole
38	Fluazifop	83	Sulfathiazol
39	Fluroxypyr	84	Sulpirid
40	Gabapentin	85	Sulpiride N-Oxide
41	Gabapentin-Lactam	86	Tramadol
42	Haloxyfop(-P) free acid		trans-10,11-Dihydroxy-10,11-dihydrocarbamazepin
43	Hydrochlorothiazide	87	
44	Indomethacin	88	Triclopyr
45	Ketoprofen	89	Trimethoprim

Figure S 2: Repeatability of extraction, A: without considering geometry of the elution head and the band geometry, B: consideration of the geometry of the elution head and the band geometry (N = 5).

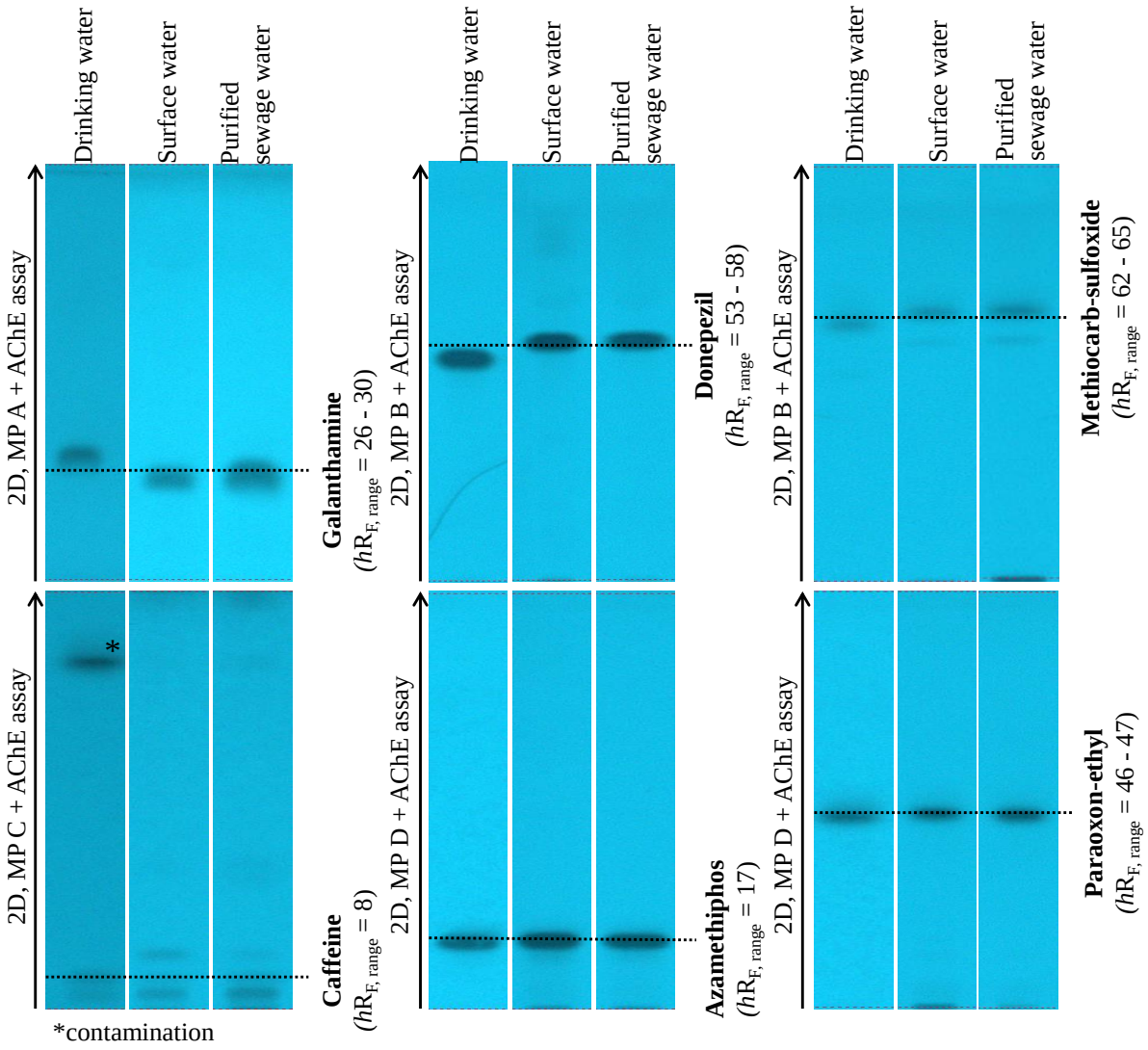


Figure S 3: Separation and AChE assay of the spiked sample extracts in the 2D

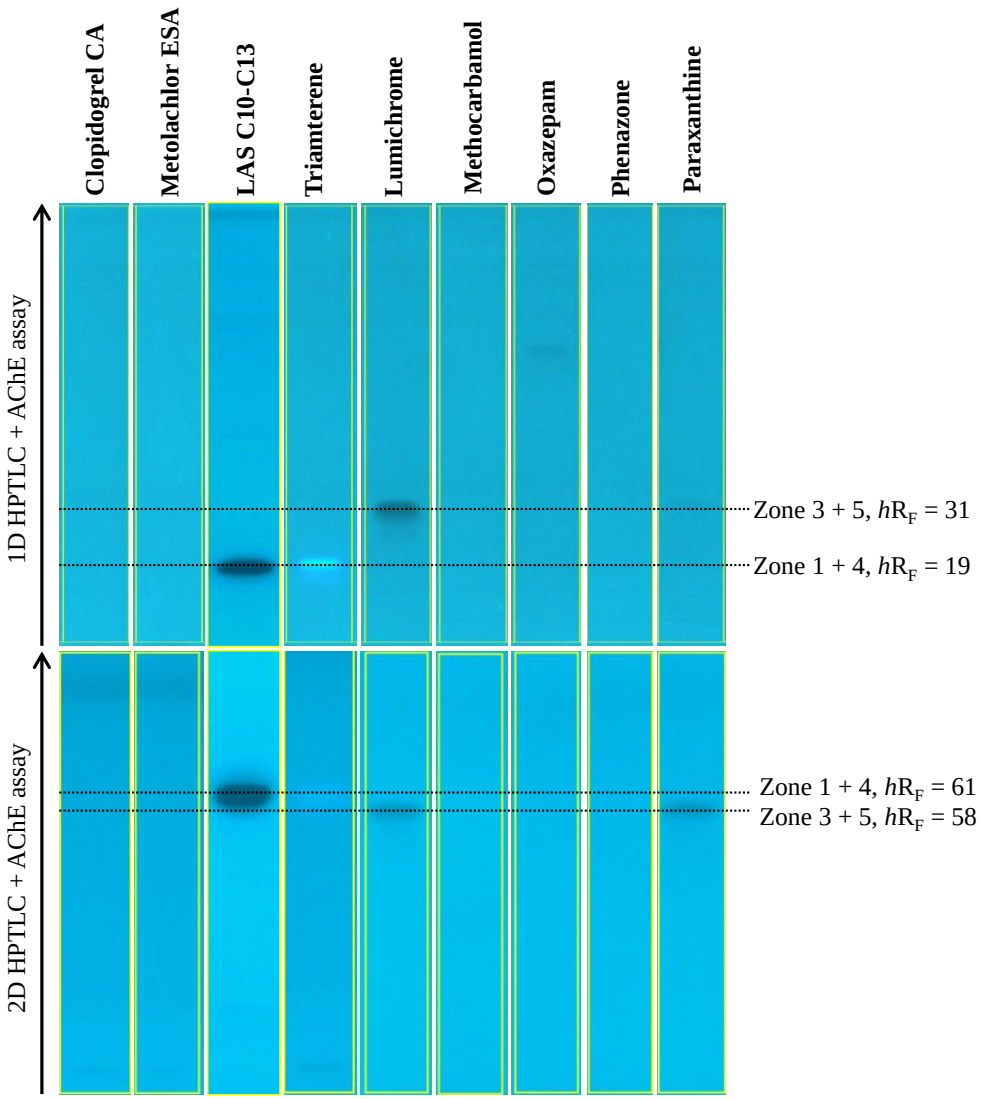


Figure S 4: Examination of effects and hR_F of the candidate substances (concentration 1000 µg/L, application volume: 10 µL)